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*Utah State University*

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# INVESTIGATION OF THE TAILORING STEPS IN PRADIMICIN BIOSYNTHESIS

by

Kandy L. Napan

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

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Logan, Utah

2016

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**ABSTRACT**

## Investigation of the Tailoring Steps in Pradimicin Biosynthesis

by

Kandy L. Napan, Doctor of Philosophy

Utah State University, 2016

Major Professors: Jixun Zhan and Jon Y. Takemoto  
Department: Biological Engineering

The actinobacteria *Actinomadura hibisca* synthesizes the natural products pradimicin A-C through a type II polyketide biosynthetic pathway. Eight tailoring enzymes in pradimicin biosynthesis have been investigated in this work, including PdmJ, PdmW, PdmN, PdmT, PdmO, PdmS, PdmQ and PdmF. PdmJ and PdmW were characterized as cytochrome P450 hydroxylases that catalyze the incorporation of two hydroxyl groups at C-5 and C-6, respectively. These enzymes worked synergistically and their co-expression significantly improved the efficiency of the hydroxylation steps. PdmN is an amino acid ligase that accepts a variety of substrates and ligates a D-alanine moiety to C-16 to form the corresponding derivatives. PdmS and PdmQ were functionally identified as *O*-glycosyltransferases. Disruption of *pdmS* in the genome of *A. hibisca* generated a biosynthetic precursor without sugar moieties, which validated that PdmS is the first glycosyltransferase that attaches the first sugar to the 5-OH of pradimicins. In contrast, disruption of *pdmQ* led to the synthesis of pradimicin B, confirming that PdmQ was responsible for attaching the D-xylose moiety to the 3'-OH of the first sugar portion in



pradimicins. Naturally, the first sugar moiety 4',6'-dideoxy-4'-amino-D-galactose of pradimicin A and B is methylated at 2'-NH. When the expression of PdmO was compromised, the mutant strain produced mainly pradimicin C, which contains the 4',6'-dideoxy-4'-amino-D-galactose in its structure. This suggested that PdmO was responsible for the *N*-methylation of the amino sugar. PdmF was identified as the C-11 *O*-methyltransferase. Moreover, PdmT was confirmed to be an *O*-methyltransferase through gene disruption and *in vitro* biochemical reactions. PdmT methylates the 7-OH to form a methoxy group that in a later step is removed to generate the pradimicin aglycon.

In summary, this research has identified eight important pradimicin biosynthetic enzymes that are involved in various tailoring steps in pradimicin biosynthesis. Several new pradimicin analogues has been generated by manipulating these enzymes. Their enzymatic properties and collaborative actions were investigated. These results not only provide new insights into type II polyketide biosynthetic pathways, but also enable rational engineering of the pradimicin biosynthetic pathway to create new analogues for drug development.

**PUBLIC ABSTRACT**

## Investigation of the Tailoring Steps in Pradimicin Biosynthesis

by

Kandy L. Napan, Doctor of Philosophy

Utah State University, 2016

Major Professors: Jixun Zhan and Jon Y. Takemoto  
Department: Biological Engineering

This research focused on the investigation of the late steps in the biosynthetic pathway of the novel antifungal and antiviral pradimicins A-C. Pradimicins were first isolated from the soil bacterium *Actinomadura hibisca*. These bioactive molecules are assembled by a type II polyketide biosynthetic pathway. Although the biosynthetic gene cluster of pradimicin has been identified, the functions of the biosynthetic genes and how they work collaboratively to form the final structures of pradimicins remain unknown. This research aims to functionally characterize the enzymes involved in the late steps of the biosynthetic route.

The early biosynthetic steps of pradimicins have been previously investigated, and the enzymes required to form the core pentangular structure have been identified. This dissertation research characterized eight biosynthetic enzymes that further tailor the core structure. These enzymes include PdmJ, PdmW, PdmN, PdmT, PdmO, PdmS, PdmQ and PdmF. Main research approaches included gene disruption, heterologous expression, combinatorial biosynthesis, and *in vitro* biochemical studies. PdmJ and PdmW were

characterized as cytochrome P450 hydroxylases that catalyze the incorporation of two hydroxyl groups at C-5 and C-6, respectively. These enzymes worked synergistically during pradimicin biosynthesis. PdmN is an amino acid ligase with broad substrate specificity. PdmS and PdmQ were identified as the dedicated *O*-glycosyltransferases that are responsible for the introduction of the first and second sugar moieties. Three methyltransferases involved in pradimicin biosynthesis were also identified. PdmO is a *N*-methyltransferase responsible for the methylation of the amino sugar moiety. PdmF was identified as the C-11 *O*-methyltransferase, while PdmT was confirmed to be an *O*-methyltransferase that methylates the 7-OH.

Engineered biosynthesis has shown its promise in creating chemical diversity for drug discovery. This research has identified eight critical biosynthetic enzymes in the biosynthesis of pradimicins, a group of promising antiviral and antifungal compounds. The results from this work provide an important knowledge and technical basis for further engineering of this pathway to generate new pradimicin analogues for bioactivity screening.

Kandy L. Napan

**DEDICATION**

I would like to dedicate my doctoral dissertation to my loving parents Francisca Molina and Pablo Napan for their always strong words of encouragement from afar. To my husband, Hao-Chieh Hsieh, for his tireless support and for his many hours spent waiting patiently and to my beloved son, Arthur Hsieh.

Kandy L. Napan

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## CONTENTS

	Page
ABSTRACT .....	iii
PUBLIC ABSTRACT .....	v
DEDICATION .....	vii
ACKNOWLEDGMENTS .....	viii
LIST OF TABLES .....	xii
LIST OF FIGURES .....	xiii
ACRONYMS .....	xvi
CHAPTER 1 .....	1
INTRODUCTION .....	1
1.1 Background .....	1
1.1.1 Antibiotic producing bacteria .....	1
1.1.2 Pradimicins .....	2
1.1.3 Carbohydrate binding agents .....	6
1.1.4 Antiviral and antifungal activity .....	7
1.1.5 Polyketide synthase (PKS) .....	14
1.2 Engineering Significance .....	20
1.3 Research Objectives .....	21
1.4 A Guide to the Dissertation .....	23
1.5 References .....	23
CHAPTER 2 .....	28
A KEY CYTOCHROME P450 HYDROXYLASE .....	28
2.1 Abstract .....	28
2.2 Introduction .....	28
2.3 Materials and Methods .....	29
2.3.1 Primers and cloning .....	29
2.3.2 Protein expression and purification .....	31
2.3.3 Spectroscopy characterization of the cytochrome P450 .....	32

2.3.4	PdmJ kinetics.....	32
2.3.5	Biotransformation.....	32
2.4	Results and Discussions .....	33
2.5	Conclusions .....	41
2.6	Acknowledgements .....	42
2.7	References .....	42

CHAPTER 3 .....	45
-----------------	----

SYNERGISTIC ACTIONS OF TAILORING ENZYMES IN PRADIMICIN BIOSYNTHESIS .....	45
--	----

3.1	Abstract .....	45
3.2	Introduction .....	45
3.3	Materials and Methods .....	48
3.3.1	General .....	48
3.3.2	Bacterial strain and vectors .....	49
3.3.3	Growth media and conditions.....	49
3.3.4	Construction of plasmids.....	50
3.3.5	<i>In vivo</i> D-alanylation .....	50
3.3.6	Extraction and isolation of compounds .....	51
3.3.7	Determination of the absolute configuration of 5 and 8.....	52
3.3.8	Antifungal assays of 4-11 .....	52
3.4	Results and Discussions .....	53
3.4.1	Characterization of PdmW as the C-6 hydroxylase .....	53
3.4.2	Synergistic actions of two CYP hydroxylases in pradimicin biosynthesis .....	57
3.4.3	Broad substrate specificity of PdmN.....	60
3.4.4	Combinatorial biosynthesis of new pradimicin analogues.....	61
3.5	Conclusions .....	66
3.6	Acknowledgements .....	67
3.7	References .....	67

CHAPTER 4 .....	69
-----------------	----

THREE ENZYMES INVOLVED IN THE <i>N</i> -METHYLATION AND INCORPORATION OF THE PRADIMICIN SUGAR MOIETIES .....	69
---	----

4.1	Abstract .....	69
4.2	Introduction .....	70
4.3	Materials and Methods .....	72
4.3.1	Primers and plasmid construction .....	72
4.3.2	Intergeneric conjugation.....	73
4.3.3	Large scale culture and purification .....	75
4.3.4	LC-MS and NMR analysis.....	75

4.4	Results and Discussion .....	75
4.4.1	BLAST analysis .....	75
4.4.2	Plasmid construction .....	77
4.4.3	Confirmation of mutants by PCR and LC-MS analysis .....	78
4.4.4	Structure determination by NMR .....	81
4.5	Conclusions .....	82
4.6	Acknowledgements .....	83
4.7	References .....	83
CHAPTER 5 .....		85
TWO PUTATIVE O-METHYLTRANSFERASES: PdmT and PdmF.....		85
5.1	Abstract .....	85
5.2	Introduction .....	86
5.2.1	Methyltransferases in natural product biosynthesis .....	86
5.3	Materials and Methods .....	89
5.3.1	General .....	89
5.3.2	Primers and cloning.....	90
5.3.3	Intergeneric conjugation approach .....	91
5.3.4	Combinatorial biosynthesis approach for <i>pdmF</i> .....	91
5.3.5	Protein expression and purification.....	91
5.3.6	Kinetics.....	93
5.3.7	Compound purification and structure determination by NMR .....	94
5.4	Results and Discussions .....	95
5.5	Conclusions .....	104
5.6	Acknowledgements .....	105
5.7	References .....	105
CHAPTER 6 .....		108
CONCLUSIONS AND RECOMMENDED FUTURE RESEACH.....		108
6.1	Conclusions .....	108
6.2	Recommended Future Research .....	109
APPENDICES .....		111
APPENDIX A.....		112
APPENDIX B. ....		140
APPENDIX C. ....		157



## LIST OF TABLES

Table	Page
1. Structures of pradimicins and their derivatives. ....	3
2. Pradimicin biosynthetic intermediates. ....	8
3. <i>In vitro</i> antifungal activity of pradimicin A, B, and C. ....	11
4. Summary of the three types of PKSs. ....	22
5. Primers designed to clone <i>pdmJ</i> and <i>gdh</i> genes. ....	30
6. Plasmids constructed in this study and corresponding polyketide products. ....	53
7. The <sup>1</sup> H NMR data (300MHz) of 8, 9 and 11 ....	56
8. The <sup>13</sup> C NMR data (75MHz) of 8, 9 and 11 ....	56
9. Primers to clone the upstream and downstream region of each gene. ....	73
10. <sup>1</sup> H and <sup>13</sup> C NMR data of 12 ....	77
11. Plasmids designed to disrupt the genes <i>pdmS</i> , <i>pdmQ</i> , and <i>pdmO</i> . ....	78
12. Designed PCR primers for knockout and protein expression. ....	90
13. Plasmids used for combinatorial biosynthesis approach for PdmF. ....	93
14. Plasmids for double cross over recombination and protein expression. ....	95
15. The NMR data of KN85 (13) and 7- <i>O</i> -methyl-KN85 (14). ....	102

## LIST OF FIGURES

Figure	Page
1. HIV-1 strain under pradimicin A pressure.....	10
2. Putative binding model of pradimicin A and Man-OMe by Nakagawa. ....	13
3. Representation of the pradimicin A/Ca <sup>2+</sup> complex by Nakagawa. ....	14
4. Representative polyketide natural products. ....	16
5. Biosynthesis of erythromycin A .....	17
6. Biosynthesis of tetracenomycin by a type II PKS. ....	19
7. Biosynthesis of flaviolin by a type III PKS. ....	20
8. Amino acid sequence alignment. ....	34
9. PdmJ and GDH expression plasmids.....	35
10. SDS-PAGE analysis of expression and purification of PdmJ. ....	36
11. Spectroscopic and enzymatic analyses of PdmJ. ....	37
12. HPLC analysis of C-5 hydroxylation by PdmJ at 460 nm.....	39
13. Synergistic actions of two CYP hydroxylases in pradimicin biosynthesis.....	55
14. Key <sup>1</sup> H- <sup>1</sup> H COSY and HMBC correlations for 8, 9 and 11.....	57
15. Determination of the absolute configurations of 5 and 8.....	58
16. HPLC analysis of alanylation by PdmN. ....	59
17. Combinatorial biosynthesis of new pradimicin analogues. ....	63
18. Biosynthetic pathway of 1. The steps studied in this work are boxed.....	64
19. The structure of the pradimicin aglycon (12). ....	71
20. HPLC analysis of the products synthesized by the mutant strains. ....	79

21.	Disruption of <i>pdmS</i> , <i>pdmQ</i> and <i>pdmO</i> in <i>A. hibisca</i> .....	81
22.	Verification of the correct mutants by PCR.....	82
23.	Visual conclusion of the last steps in the synthesis of 1.....	83
24.	Reaction mode of SAM-dependant NPMTs.....	88
25.	Structure determination of compounds 13 and 14 by NMR.....	96
26.	LC/MS chromatograms and absorbance of compounds 1, 13, and 14.....	97
27.	Construction and verification of the $\Delta pdmT$ mutant strain.....	98
28.	SDS-PAGE analysis of the expression and purification of PdmT.....	99
29.	Proposed reaction catalyzed by PdmT.....	100
30.	SDS-PAGE analysis of the expression and purification of PdmF.....	100
31.	LC/MS analysis of new pradimicin analogues.....	101
32.	LC/MS analysis of PdmF in vitro reaction.....	103
33.	Proposed methyl transfer reactions from SAM by PdmF.....	104
34.	Key $^1\text{H}$ - $^1\text{H}$ ROESY correlations for 9 and 11.....	140
35.	Determination of the absolute configurations of 5 and 8.....	140
36.	ESI-MS (-) spectrum of compounds 4-11.....	141
37.	UV/Vis spectrum of compounds 4-11.....	142
38.	$^1\text{H}$ -NMR spectrum of compound 8.....	143
39.	$^{13}\text{C}$ -NMR spectrum of compound 8.....	144
40.	$^1\text{H}$ - $^1\text{H}$ COSY of compound 8.....	145
41.	HMBC of compound 8.....	146
42.	$^1\text{H}$ -NMR spectrum of compound 9.....	147
43.	$^{13}\text{C}$ -NMR spectrum of compound 9.....	148

44.	$^1\text{H}$ - $^1\text{H}$ COSY of compound 9.....	149
45.	HMBC of compound 9.....	150
46.	$^1\text{H}$ -NMR spectrum of compound 11.....	151
47.	$^{13}\text{C}$ -NMR spectrum of compound 11.....	152
48.	$^1\text{H}$ - $^1\text{H}$ COSY of compound 11.....	153
49.	HMBC of compound 11.....	154
50.	$^1\text{H}$ -NMR spectrum of compound 14.....	155
51.	$^1\text{H}$ - $^1\text{H}$ COSY of compound 14.....	156
52.	UV/Vis spectrum of 1, 2, 3, and 12.....	157
53.	ESI-MS spectrum of 1, 2, 3, and 12.....	158

## ACRONYMS

### Cases

aa: Amino acid

ACP: Acyl carrier protein

amu: Atomic Mass unit

Apr<sup>r</sup>: Apramycin resistance

CoA: Coenzyme A

DARR: Dipolar assisted rotational resonance

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

GDH: Glucose dehydrogenase

g: Gram

GT(s): Glycosyltransferase(s)

HIV: Human immunodeficiency virus

HPLC: High performance liquid chromatography

IPTG: Isopropyl  $\beta$ -D-1-thiogalactopyranoside

LB: Luria Bertani broth

LC/MS: Liquid chromatography/Mass spectroscopy

mg: Milligram

MS: Mannitol soya flower medium

MT(s): Methyltransferase(s)

MTPA: Mosher's acid, or  $\alpha$ -methoxy- $\alpha$ -trifluoromethylphenylacetic acid

NADPH: Nicotinamide adenine dinucleotide phosphate

NCBI: National center for biotechnology information

Ni-NTA: Nickel-nitrilotriacetic acid

NMR: Nuclear magnetic resonance

*N*-MT: *N*-Methyltransferase

NRRL: Northern regional research laboratory

OD: Optical density

*O*-GT: *O*-glycosyltransferases

*O*-MT: *O*-methyltransferase

ORF: Open reading frame

PCR: Polymerase chain reaction

Pdm(s): Pradimicin(s)

PEG: Polyethylene glycol

PKSs: Polyketide synthases

PMSF: Phenylmethylsulfonyl fluoride

SAM: S-Adenosyl-L-methionine

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

tsr: Thiostrepton resistance

YM: Yeast extract/Malt extract medium

# CHAPTER 1

## INTRODUCTION

### 1.1 Background

#### 1.1.1 Antibiotic producing bacteria

Among many bacteria that produce secondary metabolites, *Actinomycetales*, produce a large variety of complex compounds synthesized by the class of enzymes polyketide synthases (PKSs). Studies have revealed that polyketide are a rich source of functional molecules, such as antibiotics of pharmaceutical application, pigments, insecticides, immunosuppressives, and others. *Actinomycetales* species grow in various environments and their morphological characteristics look like filamentous forms comparable to fungi; however, the hyphae can form a chain of spores. In this taxonomical class, the genus *Streptomyces* obtains great attention, because they are among the most adaptable and abundant soil microorganisms. They can synthesize a variety of bioactive molecules such as antibiotics, antifungals, antitumoral, antivirals, anti-hypertensives, immunosuppressives, and others. *Streptomyces coelicolor* is one of the most studied species in this group; it is a model system to study the synthesis, regulation, and metabolism of second metabolites.<sup>1</sup> Genetics studies into antibiotic regulation have been significantly enabled by the study of its pigmented metabolites: the red compound undecylprodigiosin and the blue as actinorhodin.<sup>1</sup>

The antibiotics derived from actinomycetales began with the discoveries of streptothricin (streptothricin F) in 1942 and of streptomycin in 1944, as well as a larger movement to systematically screen for antibiotics.<sup>2</sup> From 1983 to 1994, 60-80% of antibacterial and anticancer medicines were derived from natural products.<sup>3</sup> By the 1960s,

about 80% of antibiotics were isolated from the genus *Streptomyces* and it has been estimated that 294,300 antimicrobial compounds present in the genus *Streptomyces* are waiting to be isolated by 2020.<sup>2</sup>

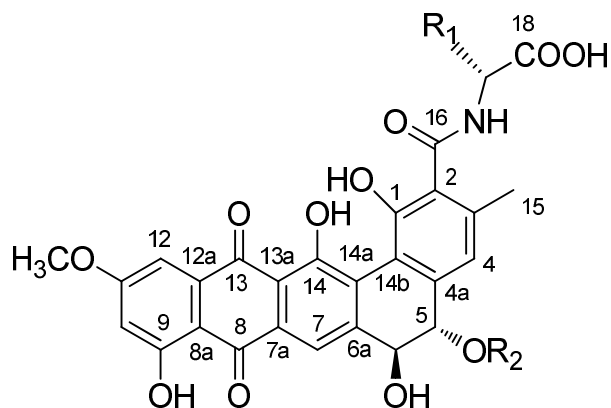
In the same taxonomic class, the species *Actinomadura hibisca*, isolated from a soil sample of the Fiji Island, produces a red pigment that protected mice from *Candida*, *Aspergillus*, and *Cryptococcus* strains.<sup>4</sup> *A. hibisca* forms a white aerial mycelium and long straight spore-chains when cultivated in YM solid medium (yeast extract, malt extract, and glucose) and ISP4 (inorganic salt starch agar). The temperature range for growing is 18 °C - 40 °C. The second metabolites or red pigments synthesized showed promising *in vitro* bioactivity against the opportunistic pathogen *Candida albicans*, *Aspergillus*, *Cryptococcus*, and *Sporothrix*<sup>5</sup> and prevent the early step of HIV infection<sup>6, 7</sup> (Table 3).

#### 1.1.2 Pradimicins

In 1988, Oki and coworkers obtained the red pigments produced by *A. hibisca* through precipitation at mildly acidic condition (pH = 5) and purified them using column chromatography. The structures of the components were determined by chemical degradation and spectral analysis and were named pradimicin A, B, and C. Their structures encloses a 5,6-dihydrobenzo( $\alpha$ )naphthacenequinone core, an amino acid (D-alanine), and a disaccharide moiety consisting of D-xylopyranose and 4-methylamino-4-deoxy-D-fucopyranose in pradimicin A. The sugar moiety of pradimicin B contains only a D-xylopyranose and in pradimicin C the second sugar is substituted by a 4-amino-4-deoxy-D-fucopyranose. The unique bioactivity and the continuous need for antifungal and antiviral treatments make this group of compounds a good candidate for synthesis studies.



Table 1. Structures of pradimicins and their derivatives.



Compound	R <sub>1</sub>	R <sub>2</sub>	Bioactivity against
Pradimicin A <sup>4</sup> ( <b>1</b> )	CH <sub>3</sub>		<i>C. albicans</i> , <i>Aspergillus</i> sp., and anti-HIV. <sup>8,9</sup>
Pradimicin D <sup>9</sup>	H		<i>Cryptococcus neoformans</i> and <i>A. flavus</i> . <sup>9</sup>
Pradimicin FA-1 <sup>13</sup>	CH <sub>2</sub> OH		<i>C. neoformans</i> and <i>Sporothrix schenckii</i> . <sup>10</sup>
Pradimicin B ( <b>2</b> ) <sup>4</sup>	CH <sub>3</sub>		<i>Saccharomyces cerevisiae</i> and <i>C. neoformans</i> . <sup>8</sup>
Pradimicin FB <sup>11</sup>	CH <sub>2</sub> OH		<i>C. neoformans</i>
Pradimicin C ( <b>3</b> ) <sup>4</sup>	CH <sub>3</sub>		<i>Sporothrix schenckii</i> . <sup>8</sup>
Pradimicin E <sup>9</sup>	H		<i>C. neoformans</i> and <i>C. albicans</i> . <sup>9</sup>
Pradimicin FA-2 <sup>13</sup>	CH <sub>2</sub> OH		<i>C. neoformans</i> and <i>S. schenckii</i> . <sup>10</sup>
Pradimicin S <sup>11</sup>	CH <sub>3</sub>		<i>S. cerevisiae</i> , <i>C. albicans</i> , <i>C. neoformans</i> , <i>Aspergillus fumigatus</i> , <i>Trichophyton mentagrophytes</i> , anti-HIV, and anti-influenza A (except pradimicin FS). <sup>11</sup>
Pradimicin FS <sup>11</sup>	CH <sub>2</sub> OH		
BMY-28864 ( <b>7</b> ) <sup>7</sup>	CH <sub>2</sub> OH		<i>C. albicans</i> and <i>C. neoformans</i> . <sup>12,13</sup>

Compound	R <sub>1</sub>	R <sub>2</sub>	Bioactivity against
N,N-Dimethyl pradimicin E <sup>13</sup>	H		<i>S. cerevisiae</i> , <i>C. albicans</i> ATCC38247, and <i>C. neoformans</i> . <sup>13</sup>
BMS-181184 <sup>14</sup>	CH <sub>2</sub> OH		<i>Candida</i> species (>10), <i>Trichosporon beigelii</i> , <i>Microsporium canis</i> , and <i>Paecilomyces varioti</i> . <sup>14, 15</sup>
Pradimicin FL <sup>11</sup>	CH <sub>2</sub> OH		<i>C. neoformans</i> , <i>S. cerevisiae</i> , and <i>A. fumigatus</i> . <sup>16</sup>
Pradimicin L <sup>11</sup>	CH <sub>3</sub>		<i>C. neoformans</i> . <sup>16</sup>

As a consequence, the exploration of other bacterial species and chemical modifications of pradimicins have involved a major effort by several groups in order to find a more water soluble pradimicin (pradimicin A  $\geq 50 \mu\text{M}$ ).<sup>17, 18</sup> More than 10 analogues are pradimicin congeners with changes in the structure of their molecules that were chemically modified, extracted from other species of *Actinomadura*, or mutants strains (Table 1). The bioactivity of the analogues of pradimicin listed in Table 1 varies within yeast, virus, and fungi species. The antiviral activity against HIV-1 inhibited its cytopathic effects on various strains with an EC<sub>50</sub> ranging from 5.2 to 5.9  $\mu\text{g mL}^{-1}$ .<sup>19</sup> Pradimicin A was not toxic to cell cultures at 50  $\mu\text{g mL}^{-1}$ , highest concentration tested due to low solubility.<sup>6</sup> Pradimicin S, had comparable EC<sub>50</sub> values ranging from 5.1 to 8.9  $\mu\text{g mL}^{-1}$ .<sup>19</sup>

The biosynthetic pathway of pradimicins has been proposed based on the deduced functions of the protein sequences found in the gene cluster of pradimicin. The pradimicin gene cluster of *A. hibisca* is 39 kb and, contains 28 open reading frames.<sup>20</sup> The proteins

involved in the pradimicin synthesis have strong similarities with the proteins in the confirmed synthesis pathway of the antibiotics griseorhodin, fredericamycin, and rubromycin.<sup>20</sup>

Current research has proven that pradimicins are naturally synthesized by a type II polyketide synthase (PKS). Like the biosynthesis of many others type II PKS, the backbone of pradimicin A was proposed to originate via Claisen condensation of acyl-CoA precursors,<sup>21</sup> i.e., one acetyl as the starter unit and 11 malonyl Co-A as the extender units. The minimal PKS is made up of the enzymes PdmA, PdmB, and PdmC,<sup>22</sup> which are ketosynthase (KS), chain length factor (CLF), and acyl carrier protein (ACP), respectively. They are responsible for formation of the linear polyketide, which later undergoes cyclization by PdmD between the C9-C14 and C7-C16 intramolecular aldol condensations in the rings A and B.<sup>23</sup>

Further studies showed that PdmA, PdmB, PdmC, PdmD, together with PdmK, PdmH and PdmL must be expressed together to form the pentacyclic core. This finding was the first evidence that monooxygenase (PdmH) and cyclases (PdmL and PdmK) work synergistically to oxidize ring B, and subsequently cyclize rings C and D.<sup>23</sup>

Additionally, PdmG is the KR responsible for the reduction of a ketone to an alcohol at C-6 (or C-19<sup>23,24</sup>), making possible the reaction of the three enzymes PdmH, PdmK, and PdmL<sup>23</sup> to form the pentangular aromatic structure. The intermediate formed undergoes reduction of the hydroxyl group at C-6 with an endogenous reductase. After the pentacyclic core is formed, PdmF is the methyltransferase responsible for catalyzing the methylation of the C-11 hydroxyl in ring A. PdmN is responsible for the amidation process, and it can accept D-serine as well as various other substrates analogues to pradimicins.<sup>24,25</sup>

The two P450 enzymes in the pradimicin gene cluster, PdmJ and PdmW, were characterized as CYP hydroxylases. They are responsible for connecting the oxygen in the C5 and C6 of ring D that did not originate from the malonyl-CoA precursor.<sup>24, 25</sup> Interestingly, these CYP enzymes did not work efficiently when expressed separately to form monohydroxylated products (**5**, **8**, **10**); also PdmJ showed substrate inhibition in the *in vitro* reactions<sup>26</sup>. However, both enzymes worked efficiently when expressed at the same time to form a dihydroxylated product (**9**). The synergy between PdmJ and PdmW prevents substrate inhibition in the synthesis of pradimicins.

The late pradimicin biosynthetic steps are catalyzed by two glycosyltransferases, PdmS and PdmQ. The first sugar moiety is introduced through PdmS, which attaches the first sugar, 4-deoxy-D-fucopyranose, to the aglycon; this sugar then undergoes further modification by most likely an aminotransferase (PdmU) that is later *N*-methylated by PdmO<sup>27</sup> to finally synthesize 4-methylamino-4-deoxy-D-fucopyranose. Consecutively, the second sugar D-xylose is *O*-linked by PdmQ to the hydroxyl at C-3' from the previous sugar<sup>27</sup>. It remains unclear how the 7-OH in the C ring is removed and the 14-OH is added. The monooxygenases PdmV and PdmM1 might be responsible for the addition of the OH at C-14. Investigation of the steps in the synthesis of pradimicins has led to the production of a series of pradimicin analogues and intermediates (Table 2), which provides important information for understanding the pradimicin biosynthetic pathway.

### 1.1.3 Carbohydrate binding agents

Pradimicin A has bioactivity against systemic fungal infections,<sup>28</sup> inhibit the entry of HIV-1 virions into TCD4+ cells,<sup>7, 17</sup> and behaves as a carbohydrate-binding agent (CBA) in the presence of calcium. CBA comes from different natural sources for example,

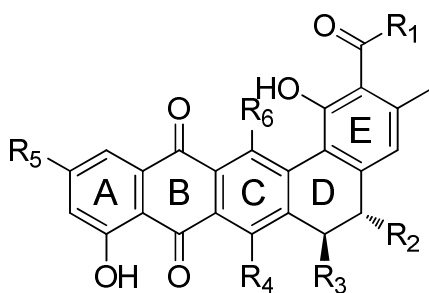
prokaryotic organisms, sea coral, algae, fungi, plants, and animals, and their nature is mostly peptidic (lectins). CBAs can be classified by their carbohydrate specificity, which may be specific to glucose, mannose, galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid.<sup>9</sup> However, little is known about non-peptidic CBAs (pradimicins). Moreover, CBAs can be classified according to their size: lectins are high molecular weight CBA and pradimicins tend to have low molecular weight.<sup>18</sup> The antiviral potency of CBAs is affected by its size: the bigger the CBAs the more variation of potency with different virus strains.

Because of pradimicin's non-peptidic nature and size, it has more advantages than peptidic CBA. Pradimicin does not show variation in potency; instead, it is consistently inhibitory against HIV-1 strains. Pradimicins can easily have contact with multiple glycan sites on the gp120 HIV molecule. Up to 33 molecules of pradimicin A has been estimated to bind a single gp120 site,<sup>6</sup> while peptidic CBAs have reduced access due to greater steric interference.<sup>15</sup> Furthermore, pradimicin A recognizes and binds selectively  $\alpha$ -1,2-mannose oligomers on the *N*-glycans from the HIV envelope gp120, which are unusual in mammalian cellular glycoproteins. Since pradimicins can easily be produced, purified, and scaled-up<sup>29</sup> pradimicin like-molecules have the potential to be used as therapeutics. The investigation for more non-peptidic CBAs must be considered.

#### 1.1.4 Antiviral and antifungal activity

Simultaneously, pradimicins are potential antifungal agents (Table 1) and antiviral small molecules.<sup>6, 17, 28, 29, 30</sup> This property is favorable to treat HIV patients whose immune system is compromised and susceptible to fungal opportunistic infections.<sup>31</sup>

Table 2. Pradimicin biosynthetic intermediates.



Analogues	R1	R2	R3	R4	R5	R6
G-2A (4)	OH	H	H	OH	OH	H
JX134 (5)	OH	OH	H	OH	OH	H
JX137a (6)		H	H	OH	OH	H
KN92 (8)	OH	H	OH	OH	OH	H
KN90 (9)	OH	OH	OH	OH	OH	H
KN102 (10)		H	OH	OH	OH	H
KN87 (11)		OH	OH	OH	OH	H
Pradimicinone I (12)		OH	OH	H	OCH <sub>3</sub>	OH
KN85 (13)		OH	OH	OH	OCH <sub>3</sub>	OH
7-O-methyl-KN85 (14)		OH	OH	OCH <sub>3</sub>	OCH <sub>3</sub>	OH
11-O-methyl-JX134 (15)	OH	OH	H	OH	OCH <sub>3</sub>	H
11-O-methyl-KN92 (16)	OH	H	OH	OH	OCH <sub>3</sub>	H
11-O-methyl-KN87 (17)		OH	OH	OH	OCH <sub>3</sub>	H

Pradimicins target *Candida*, *Cryptococcus*, and *Aspergillus* species<sup>32</sup> which are the cause of the most frequently diagnosed fungal infections. *C. albicans* frequently colonizes the oral cavity of immunosuppressed patients and adapts easily to hardship environments, eventually becoming drug resistant.<sup>32</sup>

Once HIV has infected the bloodstream, gp120 binds the macrophage receptor CD4+, undergoes a conformational change in both molecules,<sup>33</sup> and then the coreceptor CCR5 triggers endocytosis of the virus into the macrophage. By reverse transcription, the viral RNA is synthesized, forcing the cell to produce viral proteins and thus new viral particles are released from the cell through exocytosis. Eventually the gene that codes for gp120 protein is altered by mutation.<sup>34</sup> The mutated gp120 protein binds to a different co-receptor, CXCR-4 on the T-cell. The same processes occur resulting in new virus particle however, when the new viruses leave the T-cells, the viruses break the plasma membrane and kill the T-cell causing weakening of the immune system. The humoral immune system binds the free virus and virus infected cells but the immune system cannot suppress HIV because gp120 does not trigger an efficiently antibody response strong enough to neutralize the infection.<sup>35</sup> Since gp120 in HIV-1 is approximately 50% glycan, the area of the envelope surface left for antibody recognition in primary viruses is restricted.<sup>36</sup>

The mode of action of pradimicin A is based on the recognition and binding of mannose, on the fungal membrane and HIV-1 envelopes,<sup>37, 38</sup> in the presence of  $\text{Ca}^{2+}$  ions. Pradimicins are considered a non-peptidic lectin type C because it depends on  $\text{Ca}^{2+}$ , similar to the DC-SIGN lectin that originates from humans and different from GNA and HHA plant lectins that do not require  $\text{Ca}^{2+}$  to bind to gp120.<sup>6</sup> The strictly requirement of pradimicin A for  $\text{Ca}^{2+}$  to bind to highly glycosylated regions was demonstrated using

BIAcore sensor chip studies by Balzarini *et al.*<sup>6</sup> In the same fashion, pradimicin A requires  $\text{Ca}^{2+}$  for its antifungal activity to form the ternary complex between two molecules of pradimicin A, one atom of  $\text{Ca}^{2+}$ , and four molecules of mannopyranoside<sup>8, 19</sup> (Figure 3). Balzarini hypothesized that pradimicin A and other peptidic lectins have cross-linking ability that creates a mesh-like structure with immobilized surface glycoproteins, concerning the gp120 particle and the membrane of the target cell. Thus, the prevention of the virus entry to the host cells might be due to the cross-linked immobilized glycans that lose their mobility therefore their specific conformation for correct virus-cell fusion.

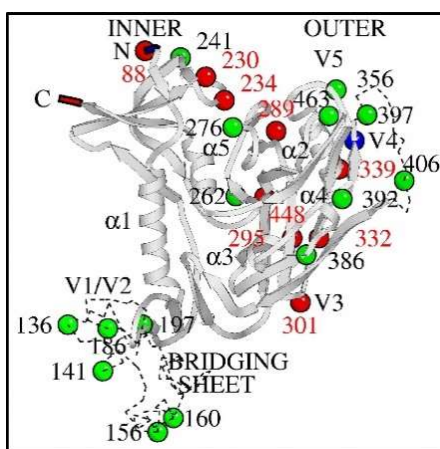


Figure 1. HIV-1 strain under pradimicin A pressure. Map of the deleted glycosylation sites in gp120<sup>6</sup>.

The pressure that pradimicin A exerts on the HIV-1 virus eventually eliminates the virus from the host after constant suppression or the virus can scape drug burden by deletions of its glycosylation sites in gp120. Figure 1 shows the results of an escalating pradimicin A exposure in CEM cell cultures, 9 (red circles) from 24 glycosylation sites in gp120 were deleted in ten virus strains. The non-mutated sites are shown in green and one site (blue) was created under pradimicin A pressure.<sup>6</sup> The deletion of the glycosylated sites



on its envelope left the virus susceptible to be neutralized and eliminated by the immune system after its glycan shield is no longer integral.<sup>36</sup> This study also reveal the molar ratio of 33 molecules of pradimicin A (840 D) and one molecule of gp120, allowing a higher number of pradimicins interacting with the virus even after mutation of few glycosylated sites. In contrast, cianovirin (11,000 D)<sup>39</sup> and HHA (50,000 D)<sup>6</sup> are peptidic anti-HIV CBAs which have a stoichiometric ratio of 1:5 and 1:1.5, gp120 to CBAs, due to its molecular weight and steric hindrance with the carbohydrates in gp120. The small size gives an advantage to pradimicin A to be a more reliable drug candidate with a higher potential to avoid pathogen drug resistance due to its high genetic barrier.<sup>29</sup>

Table 3. *In vitro* antifungal activity of pradimicin A, B, and C.

Test organism	MIC ( $\mu\text{g mL}^{-1}$ ) <sup>5</sup>		
	Pradimicin		
	A	B	C
<i>Candida albicans</i> IAM 4888	3.1	0.8	3.1
<i>Candida albicans</i> A9540	6.3	3.1	3.1
<i>Cryptococcus neoformans</i> D49	1.6	0.8	1.6
<i>Cryptococcus neoformans</i> IAM 4514	1.6	1.6	1.6
<i>Aspergillus fumigatus</i> IAM 2530	1.6	3.1	1.6
<i>Aspergillus fumigatus</i> IAM 2034	1.6	3.1	1.6
<i>Aspergillus flavus</i> FA 21436	6.3	6.3	3.1
<i>Fusarium moniliforme</i> A2284	6.3	>100	50
<i>Trichophyton mentagrophytes</i> # 4329	3.1	3.1	3.1
<i>Sporothrix Fusarium moniliforme</i> IFO 8158	0.8	3.1	0.8
<i>Petriellidium boydii</i> IFO 8158	6.3	>100	100
<i>Mucor spinosus</i> IFO 5317	>100	6.3	100

The antifungal mode of action of pradimicins is similar to their antiviral activity.<sup>5</sup>

Yanawaka studies on the interactions of pradimicin A with methyl  $\alpha$ -D-mannopyranoside and  $\text{Ca}^{2+}$  ions proposed a model for the mode of action of pradimicin A. This model exposes

the carboxylic group of D-alanine binding to mannose in the presence of  $\text{Ca}^{2+}$  ions<sup>40</sup> and the formation of a cavity that interacts with mannose; this cavity is made of the moieties of the D-alanine and the rings A, B, and C from the core of pradimicin A<sup>40, 41</sup> (Figure 2). The same ternary complex was identified for pradimicin BMY-28864, which has a D-serine as amino acid moiety and two *N*-methyl groups on its first sugar. Both pradimicins have bioactivity even though their water solubility were different, BMY-28864 is  $>20 \text{ mg mL}^{-1}$  while pradimicin A is  $0.02 \text{ mg mL}^{-1}$  in phosphate buffer saline pH 7.2.<sup>13, 42</sup>

Nakagawa and group also confirmed the geometry of pradimicin A bound to mannose by using solid-state NMR analysis (DARR technique). Their experiments successfully detected the particular close contact of D-alanine and ABC rings of pradimicin with mannans.<sup>43</sup> Also their results indicated that the C2–C4 section of mannose is situated near the primary mannan binding site in pradimicin A, the C2 carbon atom is pointing toward the ABC rings and the C3 to the D-alanine moiety. In contrast, the C1 and C6 carbon atoms face outward from the binding site. Because the C1 of mannan is directed contrary to the carboxylate group of the amino acid of pradimicin, two methyl  $\alpha$ -D-mannopyranosyl (1 $\rightarrow$ 2)- $\alpha$ -D-mannopyranoside can interact without steric interference (Figure 3). The 2-, 3-, and 4-hydroxyl groups of mannan are implicated in hydrogen bonding interactions with pradimicin A.<sup>43</sup> It is believed that the H2 proton of mannan does a CH/ $\pi$  interaction with the ring A of pradimicin A because of the short distance of 3.03 Å. It's postulated that mannan coordinates  $\text{Ca}^{2+}$  ions through the 4-hydroxyl group. Pradimicin A binds  $\alpha$ -1,2-mannose from the surface of the cell wall of yeast and  $\alpha$ -1,6-linked mannans residues which are in the backbone of cell-wall.<sup>43</sup> How pradimicin A

disrupt the cell membrane of yeast is still unknown, moreover, it seems that pradimicin A also can induce apoptosis in a mutant of *S. cerevisiae* that lack *N*-glycosylation sites.<sup>38</sup>

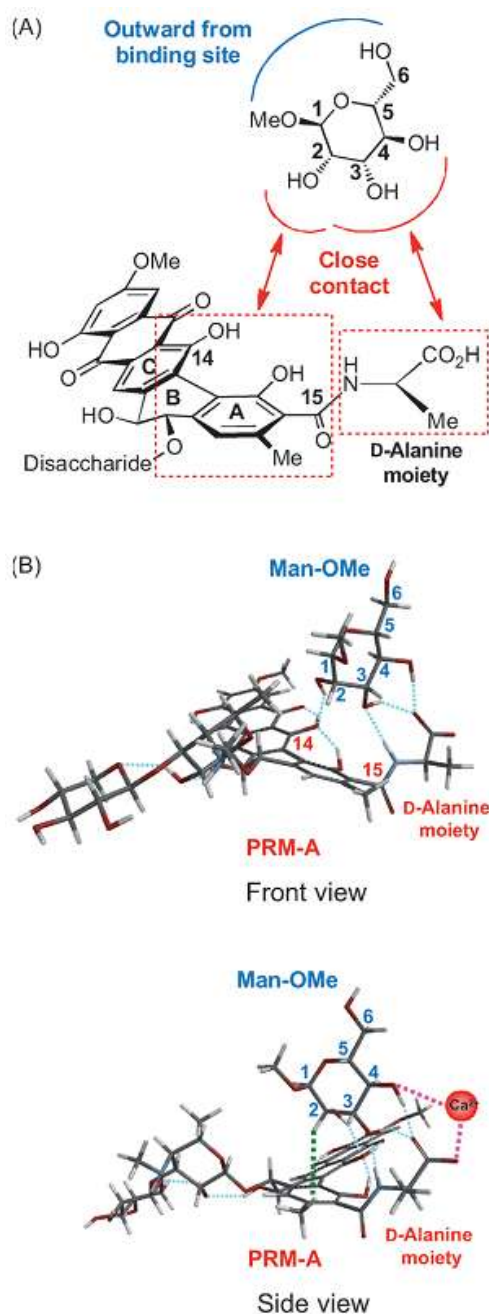


Figure 2. Putative binding model of pradimicin A and Man-OMe by Nakagawa.<sup>43</sup>

### 1.1.5 Polyketide synthase (PKS)

Polyketides are a class of secondary metabolites produced by bacteria, fungi, and plants. They are an extraordinary class of natural compounds with a broad range of functional and structural diversity. Many polyketide natural products possess medically important activities, such as antibacterial, anticancer, antifungal, antiparasitic, coccidiostats, and immunosuppressive properties (Figure 4). There are three main reasons that encourage the research on polyketides: (1) Many polyketides have the potential for drug discovery and commercial value (2). Polyketide synthases (PKSs) provide a collection of proteins, mechanism of actions, protein-protein interaction, and molecular recognition to study and develop (3). Engineering of PKSs allows the generation of novel compounds that are difficult to synthesize by other methods.

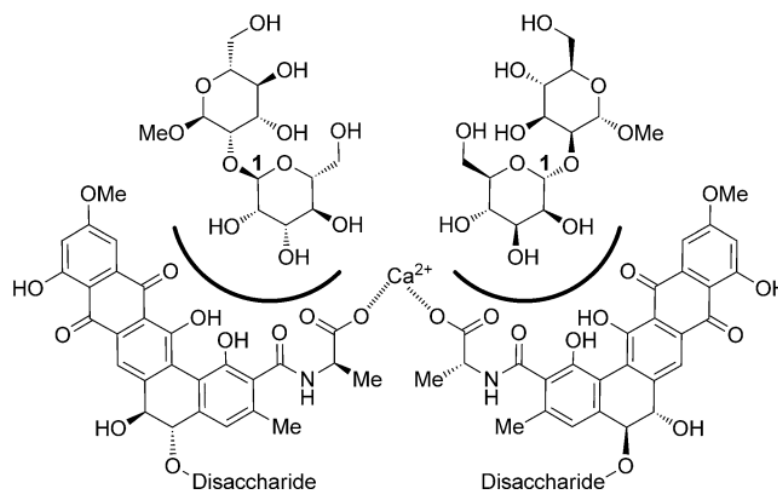


Figure 3. Representation of the pradimicin A/ $\text{Ca}^{2+}$  complex by Nakagawa. Two methyl  $\alpha$ -D-mannopyranosyl (1 $\rightarrow$ 2)- $\alpha$ -D-mannopyranoside to one molecule of pradimicin A.<sup>43</sup>

The first descriptions of bacterial type I PKSs were in 1990,<sup>44, 45</sup> type II PKSs in 1984,<sup>46</sup> and type III PKSs in 1999.<sup>47</sup> The PKS patterns have served the scientific

community by providing the molecular basis to explain the complicated structural assembly of polyketide natural products. All the PKSs known biosynthesize polyketides by consecutive decarboxylative condensation of the acyl-CoA precursors, and the KS subunit (for type II and III PKSs) catalyzes the formation of C–C bonds or the KS domain for type I PKSs.<sup>48</sup>

Type I PKSs are synthesized by bacteria and fungi. PKSs I are multifunctional polypeptides that are organized into modules which contain diverse catalytic domains. The three essential domains for chain elongation are KS, acyl transferase (AT), and ACP. Other domains such as ketoreductase (KR), enoyl reductase (ER), dehydratase (DH), and methyltransferase (MT) may be present in the PKS to form diverse polyketide structures. The domains can be used several times in fungal iterative type I PKSs to synthesize the polyketide chain. In contrast, type I PKSs from bacteria are often noniterative. In an iterative PKS, the KS domain can take back the growing polyketide chain after each condensation and terminate the polyketide biosynthesis at the proper chain size.<sup>49</sup> LovB from the biosynthetic pathway of lovastatin, a famous cholesterol-lowering drug from *Aspergillus terreus*, is an example of the fungal iterative PKS.<sup>50</sup>

In noniterative modular PKSs, each module is used once, and then the polyketide intermediate is carried to a downstream module for further chain elongation. The antibiotic erythromycin is synthesized by a bacterial noniterative type I PKS consisting of a loading module and six extension modules.<sup>51, 52, 53</sup> The precursors of erythromycin A consists of one starter unit of propionyl-CoA and six extender units of (2S)-methylmalonyl-CoA (carboxylated propionyl-CoA). The erythromycin PKS or DEBS for 6-deoxyerythronolide B synthase is made of three proteins called DEBS 1, 2, and 3. The PKS contains a loading

module, six chain extension modules, and a chain terminating thioesterase (TE). The chain elongation modules contain a KS, an AT, and an ACP domain, and variable numbers of dehydratase (DH), enoyl reductase (ER), and KR. After the TE domain releases the aglycon 6-deoxyerythronolide B, it is hydroxylated and glycosylated two times and methylated on a sugar moiety to synthesize the final structure of the antibiotic erythromycin A (Figure 5).

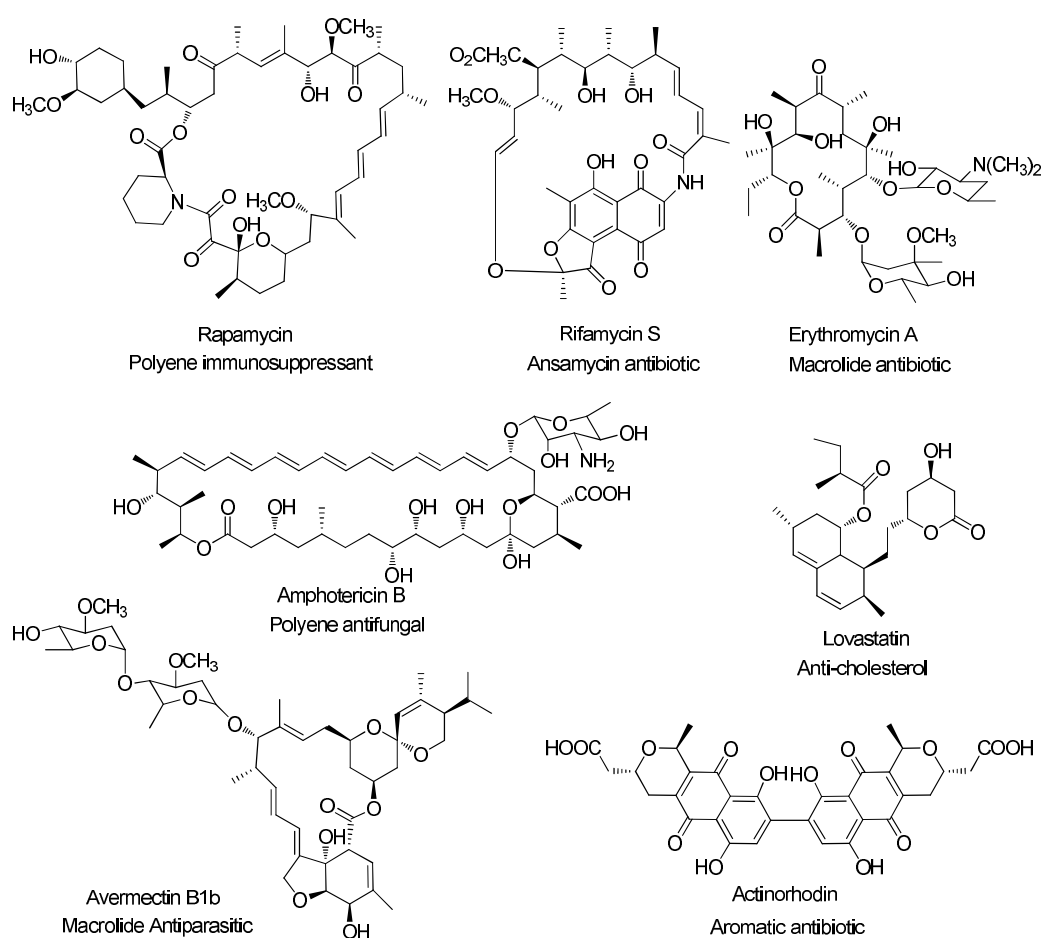
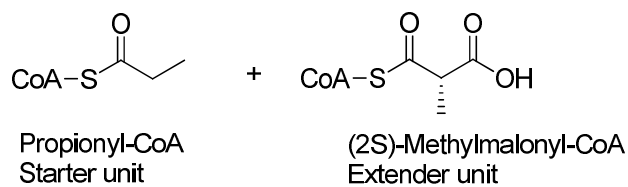


Figure 4. Representative polyketide natural products.

### Precursors



### PKS type I assembly

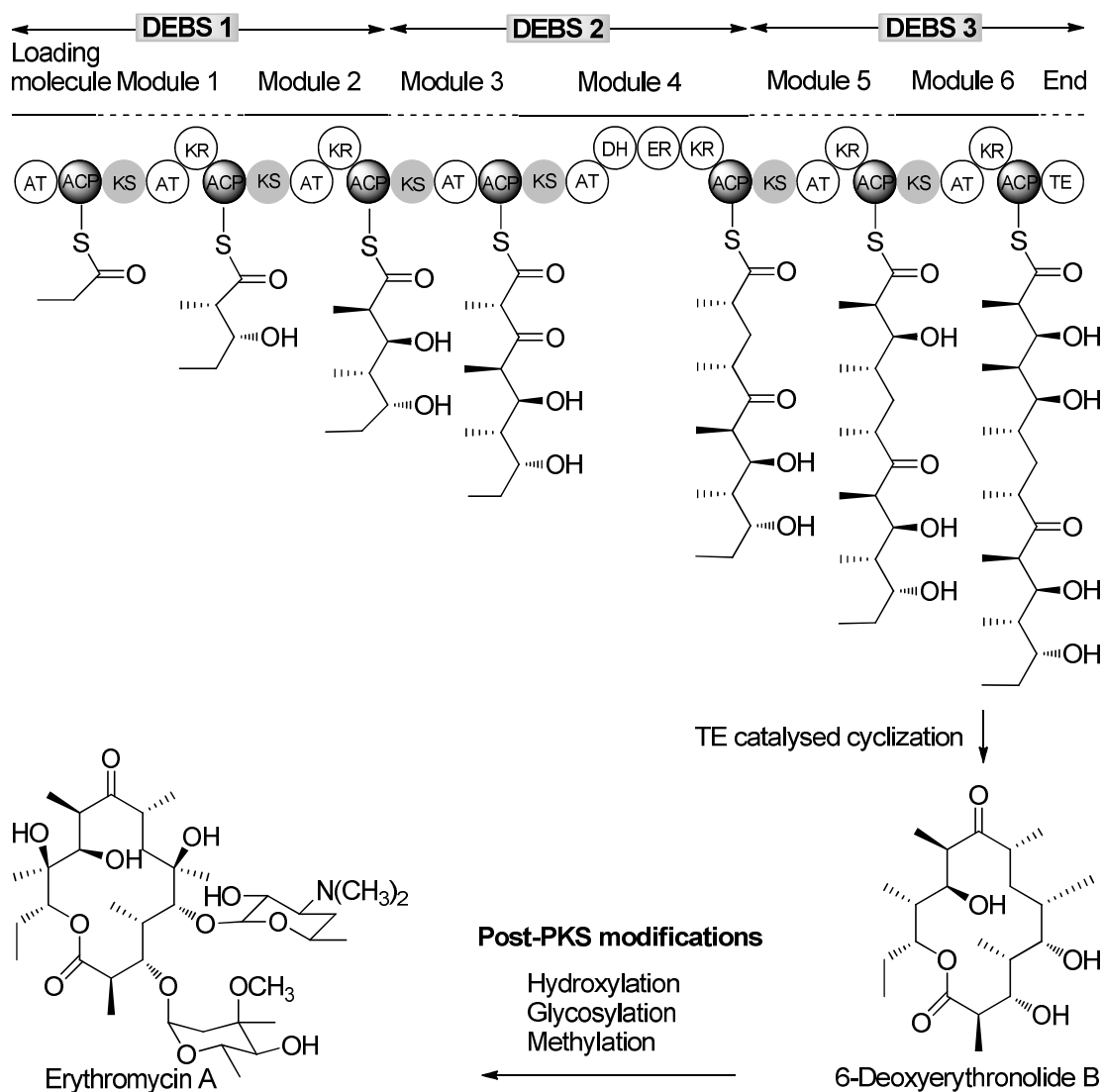


Figure 5. Biosynthesis of erythromycin A through a noniterative type I PKS pathway.

Type II PKSs are multienzyme complexes of small, discrete enzymes with specific functions. This diverse group of PKSs is essentially involved in the biosynthesis of promising drug candidates of bacterial aromatic polyketides,<sup>54</sup> such as oxytetracycline<sup>55</sup> and pradimicin.<sup>23, 25, 26</sup> The minimal PKS of type II PKSs is composed of ketoacyl synthase  $\alpha$  ( $\text{KS}_\alpha$ ), ketoacyl synthase  $\beta$  ( $\text{KS}_\beta$  or CLF) and acyl ACP. The minimal PKS synthesizes the nascent poly- $\beta$ -ketone chain to the correct length. Then the polyketide chain is exposed to alterations catalyzed by tailoring enzymes such as KR, cyclases (CYCs), MTs, glycosyltransferases (GTs), hydrolases, monooxygenases, and aminotransferases. The polyketide backbone is synthesized by condensation of an acetyl starter unit and multiple malonyl-CoA as extender units. Malonyl-CoA is also a common starter unit, as well as other CoA substrates, such as fatty acyl-CoAs and benzoyl-CoA. The structural diversity and unique biological activities of aromatic polyketides are enhanced by this common feature. In addition, CYCs enzymes contribute to the chemical diversity by forming characteristic angular, linear, or discoid aromatic ring structures. The structures are then modified by reduction, oxidation, methylation, and glycosylation to generate the final polyketide. The mentioned synthetic steps provide a wide collection of structural diversity to the polyketides. Tailoring steps of type II PKS of the antibiotic tetracenomycin C, produced by *S. glaucescens*, involves two oxygenation and three consecutive *O*-methyltransferases reactions.<sup>56</sup> (Figure 6)

Type III PKSs are small dimeric proteins (40-45 kDa) that typically undergo iterative condensation reactions with malonyl-CoA; the numbers of condensation can go from one to seven.<sup>57</sup> In spite of their simplicity, type III PKSs produce several



arrangements of metabolites such as pyrones, chalcones, phloroglucinols, acridones, resorcinolic lipids, and stilbenes.<sup>58</sup>

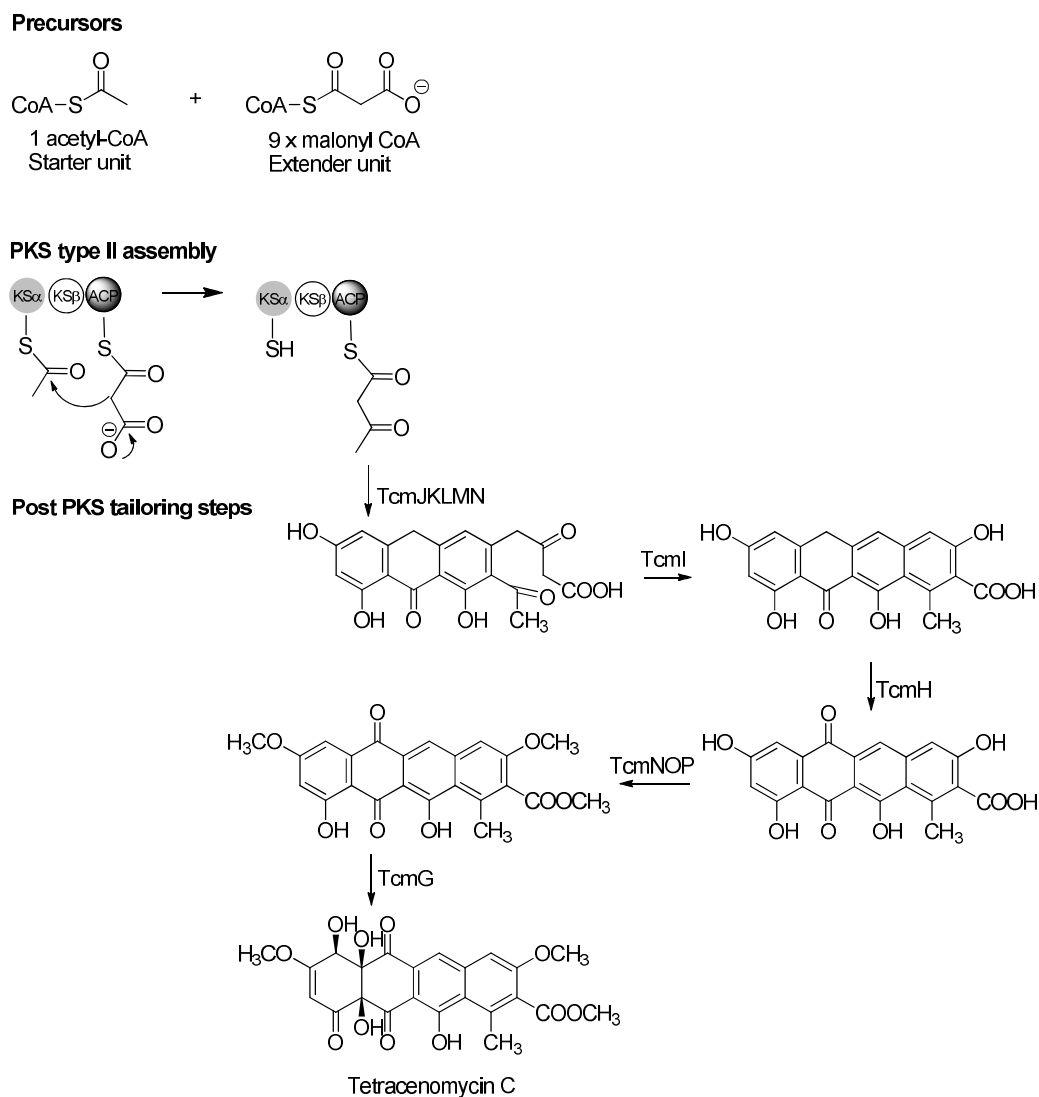


Figure 6. Biosynthesis of tetracenomycin by a type II PKS.

It was thought previously that type III PKSs were plant-specific, but the last few years have shown that bacteria and fungi also harbor type III PKSs. Five groups of bacterial type III PKSs have been described based on the structures of their outputs.<sup>58</sup> The first

bacterial type III PKS is well exemplified by RppA from the bacterium *Streptomyces griseus*.<sup>47</sup> It synthesizes 1,3,6,8-tetrahydroxynaphthalene (THN) from five molecules of malonyl-CoA, which is spontaneously oxidized to flaviolin (Figure 7). Other examples of type III PKSs are for resveratrol<sup>59</sup> and curcuminoids.<sup>41</sup> A more detailed description of the three types of PKS is shown in Table 4.

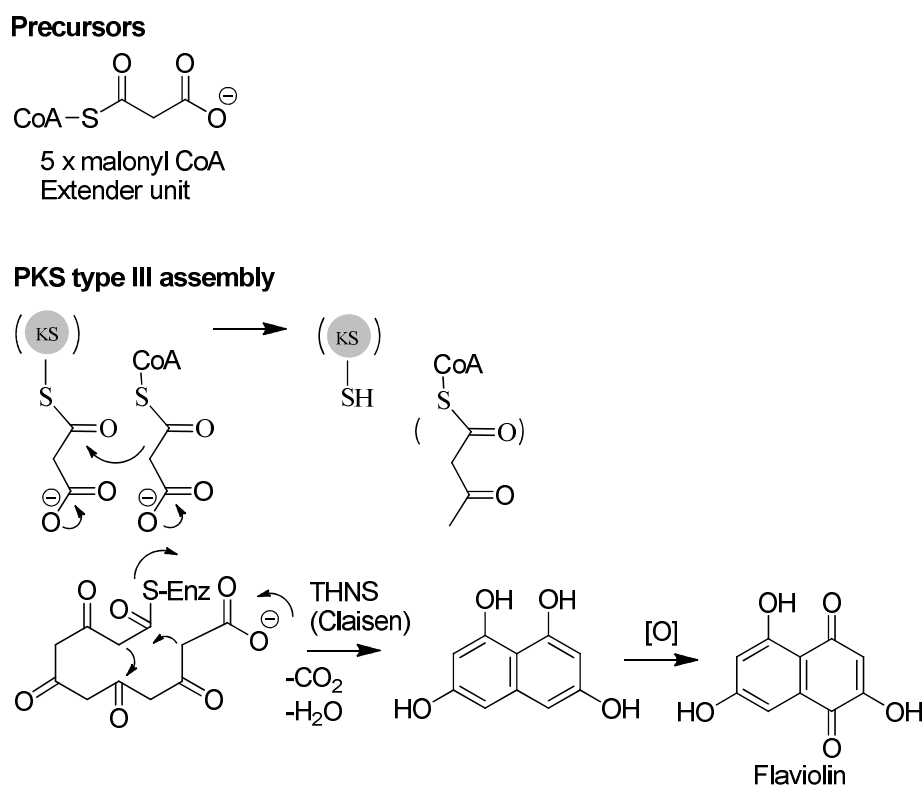


Figure 7. Biosynthesis of flaviolin by a type III PKS.

## 1.2 Engineering Significance

Biological engineers create, design, and develop feasible methods and strategies to obtain alternate and reliable therapeutics that enhance human health. With modern technology to sequence DNA, the genomic information stored in databases is challenging

researchers to process it and to understand how parts of the genome works together. Metabolic engineering could play an important role in selecting the new genetic data, understanding the functions and interactions of genes, and analyzing the innate metabolic pathways. This is applicable especially to the genetic information that synthesizes natural products with promising bioactivity. Using metabolic engineering to mimic the synthesis of therapeutic drugs in well-known microorganisms have many applications. Also, new drugs based on natural products can be synthesized by changing specific and/or using proteins or domains with specific purposes. Pradimicins have potential antifungal and antiviral properties, which metabolic pathways need to be characterized in order to further work on the synthesis of new beneficial compounds. The impact of this study in the pharmaceutical industry could have significant implications. This study decoded the last steps in the synthesis of pradimicins and produced new intermediates that allowed us to better understand the pradimicin metabolic pathway. In addition, all the accomplishments obtained in this study could have a positive impact on society because all the efforts made have provided new information on potential therapeutics for current diseases.

### **1.3 Research Objectives**

The central hypothesis for this research is that the pradimicin A biosynthetic pathway can be reconstituted and engineered in a heterologous host. The main goals of my doctoral dissertation research are to 1) decipher the biosynthetic pathway of pradimicin, with a focus on the late tailoring steps; 2) generate new pradimicin analogues using biosynthetic and biotransformation approaches. Specific objectives include:

1. Characterize the tailoring enzymes PdmJ, PdmW, PdmN, PdmS, PdmQ, PdmO, PdmF and PdmT, and determine their roles and interactions in the biosynthetic process.
2. Find and identify the intermediates in the late pradimicin biosynthetic pathway.

Table 4. Summary of the three types of PKSs.<sup>45, 49, 60, 61</sup>

	Organization	Subclasses		Minimal domains/enzymes
Type I PKS	Multi-domain enzyme. Several active sites that catalyze several cycles. Many choices of monomers.	Iterative mono-modular	Non reducing	KS, AT, PT, ACP
			Partially reducing	KS, AT, KR, DH, ACP
			Highly reducing	KS, AT, KR, DH, ER, ACP
		Non-iterative multi-modular	cis-AT	KS, AT, ACP
			trans-AT	KS, ACP, AT
Type II PKS	Iterative chain elongation by monofunctional enzymes. Tailoring enzymes.			<b>KS<sub>α</sub>, KS<sub>β</sub>, ACP</b>
Type III PKS	Simple homodimers of ketosynthases. Various reactions in one active site. Specificity of starter and extender units, length, and cyclization reactions.			
Ketosynthase (KS), acyltransferase (AT), product template domain (PT), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), ketosynthase (KS <sub>α</sub> ), chain length factor (KS <sub>β</sub> ), discrete enzymes are in bold.				

## 1.4 A Guide to the Dissertation

This dissertation is separated into six chapters. Chapter 2, published in the journal of *Bioorganic & Medicinal Letters* in 2012, will focus on the characterization of a cytochrome P450 (CYP) gene, *pdmJ*, found in the gene cluster of pradimicin. Moreover, the heterologous co-expression of *pdmJ* with glucose 1-dehydrogenase (GDH) gene was studied. Chapter 3, published in *ChemBioChem* in 2014, presents the synergistic actions of three tailoring enzymes in pradimicin biosynthesis. These three enzymes involved two CYP enzymes, *pdmJ* and *pdmW*, and one amino acid ligase, *pdmN*. PdmW was characterized as a CYP hydroxylase and its functional role in the biosynthetic pathway was determined. PdmN was characterized as a flexible amino acid ligase that receives different amino acid donors and acceptors. Chapter 4, published in the journal of *Bioorganic & Medicinal Letters* in 2015, presents the steps in the glycosylation of pradimicin A-C as well as the *N*-methylation in one of the sugar functional groups. The genes involved in these concerted steps, *pdmS*, *pdmQ*, and *pdmO*, were functionally characterized in the biosynthetic pathway of pradimicin A-C. Chapter 5 describes the characterization of two putative *O*-methyltransferases in the pradimicin pathway. Finally, chapter 6 includes a conclusion chapter that summarizes all of our findings and gives suggestions on the possible research directions.

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## CHAPTER 2

### A KEY CYTOCHROME P450 HYDROXYLASE

#### IN PRADIMICIN BIOSYNTHESIS<sup>1</sup>

##### 2.1 Abstract

Pradimicins A-C (**1–3**) are a group of antifungal and antiviral polyketides from *A. hibisca*. The sugar moieties in pradimicins are required for their biological activities. Consequently, the 5-OH that is used for glycosylation plays a critical role in pradimicin biosynthesis. A cytochrome P450 monooxygenase gene, *pdmJ*, was amplified from the genomic DNA of *A. hibisca* and expressed in *E. coli* BL21(DE3). PdmJ introduced a hydroxyl group to G-2A (**4**) at C-5 to form JX134 (**5**). A D-Ala-containing pradimicin analogue, JX137a (**6**) was tested as an alternative substrate, but no product was detected by LC-MS, indicating that PdmJ has strict substrate specificity. Kinetic studies revealed a typical substrate inhibition of PdmJ activity. The optimal substrate concentration for the highest velocity is 115  $\mu$ M under the test conditions. Moreover, the conversion rate of **4** to **5** was reduced by the presence of **6**, likely due to competitive inhibition. Coexpression of PdmJ and a glucose 1-dehydrogenase in *E. coli* BL21(DE3) provides an efficient method to produce the important intermediate **5** from **4**.<sup>1</sup>

##### 2.2 Introduction

Pradimicins A (**1**), B (**2**), and C (**3**) (Table 1) are a group of benzo[ $\alpha$ ]naphthacenequinone natural products isolated from *A. hibisca* P157-2 as antifungal

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antibiotics.<sup>2</sup> Other analogues such as pradimicins D and E were also isolated from actinomycetes.<sup>3, 4, 5, 6</sup> The structure of **1** contains an amino acid D-Ala, two sugar moieties including d-xylose and 4',6'-dideoxy-4'-methylamino-d-galactose, and a dihydrobenzo[ $\alpha$ ]naphthacenequinone aglycon. This unique structure makes **1** an effective viral entry inhibitor,<sup>7, 8</sup> and a broad-spectrum fungicide.<sup>9</sup> The sugar moieties attached to C-5 are found essential for the biological activities of **1**. Consequently, C-5 hydroxylation is a key step in pradimicin biosynthesis by providing the 5-OH for the introduction of the 4',6'-dideoxy-4'-methylamino-D-galactose moiety. Pradimicins belong to a class of natural products called polyketides. Similar to fatty acid biosynthesis, polyketides are assembled through stepwise condensations of simple carboxylic acid precursors catalyzed by enzymes known as PKS.<sup>10</sup> Three types of PKSs have been identified and designated as types I, II and III. Pradimicins are synthesized through a type II PKS pathway and the pradimicin biosynthetic gene cluster from *A. hibisca* P157-2 has been reported.<sup>11</sup> We have previously investigated the early biosynthetic steps that are involved in the formation of the pentangular polyphenol structure. The minimum set of enzymes required to afford the benzo[ $\alpha$ ]naphthacenequinone core structure includes PdmABCDHKL. Addition of PdmG led to the biosynthesis of a key intermediate G-2A (**4**).<sup>12</sup> This intermediate is subjected to a series of modifications by other tailoring enzymes to afford the final structure of **1**.

## 2.3 Materials and Methods

### 2.3.1 Primers and cloning

*A. hibisca* P157-2 was cultured in YM medium for 5 days, after which the genomic DNA was extracted from the mycelia. The gene *pdmJ* (1,236 bp, GenBank ID: EF151801)

was cloned from the genomic DNA *via* PCR using Phusion high-fidelity DNA polymerase and a pair of primers (Table 5). The start and stop codons are bold and the introduced restriction enzyme sites are underlined. The PCR program used for cloning of *pdmJ* included an initial denaturation step of 5 minutes at 98°C, a 20-cycle touch-down program (30 s denaturation at 98°C, 40 s annealing at temperatures reduced by -0.5°C/cycle from 75°C to 65°C, 80 s extension at 72°C), followed by a final extension of 10 minutes at 72°C. The PCR product was ligated into pJET1.2 between the NdeI and EcoRI sites to yield pZJ41. After sequencing, the gene was excised from pZJ41 by NdeI and HindIII and subsequently ligated into pACYCDuet-1 between the same sites, yielding pKN36 (Figure 9).

Table 5. Primers designed to clone *pdmJ* and *gdh* genes.

Primer	Sequence
<i>pdmJ</i> -F-NdeI	5'-AACATATG <b>CCCTCCTCGAAGGATGC</b> -3'
<i>pdmJ</i> -R-EcoRI	5'-AAGAATT <b>CCTACCAGGCGATGGGCAGCG</b> -3'
<i>gdh</i> -F-NdeI	5'-AACATATG <b>TATCCGGATT</b> TAAAAGG-3'
<i>gdh</i> -R-XhoI	5'-AACTCGAG <b>TTAACCGCGGCCTGCCTGGA</b> -3'

The plasmid pET20b-*gdh* that carries the gene *gdh* (786 bp) from *Bacillus subtilis* was a gift from Prof. Jianhe Xu (East China University of Science and Technology). This plasmid was used as a template to clone *gdh* using a set of primers (Table 5). The PCR program included an initial denaturation step of 5 minutes at 98°C, a 20-cycle regular program (30 s denaturation at 98°C, 40 s annealing at 62°C, 45 s extension at 72°C), followed by a final extension of 10 minutes at 72°C. The PCR product was ligated into the pJET1.2 cloning vector to yield pKN13. The *gdh* gene was excised from pKN13 by NdeI

and XhoI. Both *gdh* and *pdmJ* were subsequently ligated into pACYCDuet-1 between NdeI and XhoI as well as NcoI and HindIII, respectively, yielding pKN29 (Figure 9).

### 2.3.2 Protein expression and purification

The plasmids pKN36 and pKN29 were separately transformed into the *E. coli* BL21(DE3) competent cells using the MicroPulser electroporation apparatus (BioRad). The cells were then plated on LB agar containing 12.5 µg/mL chloramphenicol and incubated at 37°C overnight. To purify PdmJ, *E. coli* BL21(DE3)/pKN36 was grown in 500 mL of LB medium supplemented with 12.5 µg/mL chloramphenicol at 37°C on a rotary shaker at 250 rpm until the OD<sub>600</sub> reached 0.6. The broth was then induced by 200 µM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was maintained at 28°C for an additional 16 h. The cells were then pelleted by centrifugation at 4,000 rpm for 10 minutes and resuspended in the lysis buffer containing 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 1 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted by sonication on ice. Cell debris was removed by centrifugation at 20,000 rpm at 2°C for 30 min. The protein was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) column and successively eluted with buffer A (50 mM Tris-HCl, pH 7.9, 2 mM EDTA, 2 mM DTT and 0.1 µM PMSF) and buffer A containing 25 mM imidazole. The fractions were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the fraction containing PdmJ was concentrated and buffer-exchanged into buffer A using an Amicon® Ultra-15 Millipore centrifugal filter device. The purified protein was stored with 20% glycerol at -80°C for enzymatic studies.

### 2.3.3 Spectroscopy characterization of the cytochrome P450

Absorption spectra of PdmJ were recorded with a spectrophotometer within 350 - 650 nm in a quartz cuvette of 1 cm path length. 5  $\mu$ M PdmJ, diluted in 50 mM buffer Tris-HCL (pH 7.9), was added with 5 mM dithionite and then separated into 2 cuvettes. For one cuvette, the carbon monoxide complex of PdmJ was generated by bubbling CO gas into the dithionite-reduced enzyme solution (for 1 min.) at room temperature and immediately read in the spectrophotometer. The other cuvette was read within the same spectra and used as the oxidized form of PdmJ<sup>13</sup>.

### 2.3.4 PdmJ kinetics

The 100- $\mu$ L reaction mixture contained 100 mM Tris-HCl buffer (pH 7.9), 10  $\mu$ g of spinach ferredoxin, 0.05 U spinach ferredoxin-NADP reductase, 1.5 mM NADPH, 133  $\mu$ g of PdmJ, and 200  $\mu$ M **4**. The reaction was started by adding NADPH at 28°C and quenched after 10 minutes with 50  $\mu$ L of methanol. The reaction was analyzed on an Agilent 1200 LC/MS with a C18 Eclipse Plus column (5  $\mu$ m, 4.6  $\times$  250 mm), eluted with a linear gradient (10 to 90% acetonitrile in water containing 0.1% trifluoroacetic acid over 25 min).

### 2.3.5 Biotransformation

Overnight seed cultures of the strains *E. coli* BL21(DE3)/pKN36, *E. coli* BL21(DE3)/pKN29, and *E. coli* BL21(DE3)/pACYCDuet-1 were prepared. Each culture was inoculated to 50 mL LB broth supplemented with 25 mg mL<sup>-1</sup> chloramphenicol and induced with 0.2 mM IPTG and then maintained at 28°C. After 3h of induction, the substrate **4** was added to each fermentation broth and cultured for an additional 30 h. The culture was centrifuged and LC-MS analysis of the methanol extract of the sedimented and

ethyl acetate extract of the supernatant fraction were recovered. *E. coli* BL21(DE3)/pACYCDuet-1 was the control strain.

## 2.4 Results and Discussions

PdmJ and PdmW are two putative cytochrome P450 (CYP) monooxygenases found in the pradimicin biosynthetic gene cluster. CYP enzymes are widely distributed in various organisms. They play important physiological roles in the oxidative metabolism of endogenous and exogenous compounds, and are also frequently found in natural product biosynthetic pathways. Through combinatorial biosynthesis approach, PdmJ and PdmW were characterized as C-5 hydroxylase and C-6 hydroxylase, respectively. Coexpression of PdmJ with PdmABCDHKL in *S. coelicolor* CH999 gave rise to the C-5 hydroxylated product of **4**, JX134 (**5**) (Table 2). Similarly, a C-6 hydroxylated derivative of **4**, JX152, was generated when PdmW was introduced. However, the yields of both hydroxylated products were low,<sup>14</sup> which has hampered further work on other downstream tailoring enzymes. Moreover, no *in vitro* studies have been conducted to understand these key biosynthetic enzymes. In this study, we report the reconstitution of PdmJ in *E. coli* BL21(DE3) and biochemical characterization of this enzyme as the C-5 hydroxylase. The purified enzyme can efficiently convert **4** into **5** both *in vitro* and *in vivo*.

The deduced protein product of *pdmJ* has 411 amino acids. BLAST analysis of the sequence revealed numerous CYP monooxygenases, among which PdmJ shows the highest homology to LlpOVI with 57% identity and 71% similarity. The sequence alignment of PdmJ with LlpOVI, PdmW and PikC is shown in Figure 8. LlpOVI is a putative CYP monooxygenase that is involved in lysolipin biosynthesis in *S. tendae* Tü 4042.<sup>15</sup> PikC is a

CYP hydroxylase from the methymycin/pikromycin biosynthetic pathway in *S. venezuelae*,<sup>16</sup> while PdmW is another CYP hydroxylase in the pradimicin biosynthetic pathway that is responsible for C-6 hydroxylation.<sup>14</sup> As shown in Figure 8, several typical conserved motifs in CYP enzymes were identified (boxed) in PdmJ. Motif 1 G-X-[DEH]-T represents an oxygen-binding site and point of access for an incoming dioxygen molecule.<sup>17, 18</sup> Motif 2 E-X-X-R, which is conserved in all CYP enzymes, is also present in PdmJ. This motif is proposed to participate in both the redox partner interaction and heme binding.<sup>18</sup> Motif 3 represents the CYP cysteine heme-iron ligand signature [FW]-[SGNH]-X-[GD]-[F]-[RKHPT]-[P]-C-[LIVMFAP]-[GAD] as indicated in the PROSITE database (Accession number PS00086).<sup>19</sup> All these features strongly suggested that PdmJ is indeed a CYP monooxygenase.

PdmJ	1	MPSSKDAPTVDPRPDVTPAFPFRPDDPFQPPCEHARLRASDFVAKVVLPTGDHAWVVTRYADVRFVTSDRRFSKEAVTRPGAPRLIPMQ	89
LlpOVI	1	MSTEDGVRHFPFAASDPVEPPDECAALLREEPVARVRIPTGDQVWLVRHEDVRRVLSDFRFSREAITAPGAPRLPIA	79
PdmW	1	METTLPYPFRPSALEPPAELRGLR-EQPVVKVVLPSGDEAWVTRYADVRRALLADDRI SRN-LNRPGAARISAKN	75
PikC	1	MRRTQQGTTASPPVLDL GALGQDFAADPYPTYARLR AEGPAHRVRTPEGDEVVLVVG YDRARAVLADPRFSKD-WRNSTTPLTEAEA	86
PdmJ	90	RGSKS-LVIMDPPEHTMRKIVSRAFTARRVEGMRHVRDLTSGFVDEMVEHGPP-ADLIAHLALPLPVTVICEMLGVPPEDRPRF	171
LlpOVI	80	EGSKS-IFVMDPPEHSRLRRLVSRAFSPRIE SLREDVRQLAEDLV DAMVDQGP-ADLIAGLAQPLPITVICRMLGVPEYEDVERF	163
PdmW	76	KLFQDPKMDPPPEHTIRRLVMKALTPSRVERLRPRVQEITDRLVTAMAERRPP-IDVNEALAYPLPIQVVCCELLGVPEVDQERF	160
PikC	87	ALNHN-MLESDDPRHTRLKLVAREFTMRVELLRPRVQEIVDGLVDAMLAAPDGRADLMESLAWPLPITVISELLGVPEPDRAAF	171
motif 1			
PdmJ	172	QDWTDRMLTIGAPALAQADEIKAAVGRRLRGYLAELIDAKTAAPADDLLSLSRAHAD-DG--LSEEEELLTFGMTLLAAEYHTTTAA	256
LlpOVI	164	REWTDVMLS YARDGREQ---VAAARDLSGGLYTELIDAKRAHPTDDLMLVLITAADDGDR--LDDAELLAFGYTLLGAEYHATTAG	244
PdmW	161	RVWTGAFLSISGYTQDE---IERNMSRLNEYMRDLIEAKRADPDDLISAMIAVTD AEDGR-LSEYELHWSRLVLVGFETTA VQ	242
PikC	172	RVWTD A FVPDDPAQAQ-----TAMAEMSGYLSRLIDSKRGQDGEDLLSALVRTSDE-DGSRLTSEELLGMAHILLVAEHETT VNL	251
motif 2			
PdmJ	257	ITHSVYHLLREPSRYARLRDPSGIPAAVEELLRYGQIGGGAGAIRI AVEDVEVGGLVVRAGEAVIPLFNAANRDPEVFADPEELD	342
LlpOVI	245	LVHALLALVRRPADLAALDRPELMP TAVDELLRRSQAGSGLGAMRI ALEDVEIHGVRIAAGDAVL PWINAANRDPRVFD DDPDGLL	330
PdmW	243	IGGCIAMLLSRPDQLDLVRKDPALLRPAVEELLRNKIVGSSVTMLRYVTE DVEVGGV TI PAGSSI IPAGDSANQDESVEHEPEVFD	328
PikC	252	IANGMYALLSHPDQLAALRADMTLLDGAVEMLRY-EGPVESATYRFPVEPVDLDGTVIPAGDTVLVVLADAHRTPERFPDHRFD	336
motif 3			
PdmJ	343	LGRTDNPHIALGHGIHYCLGAPLARLELQVVL ETVERTPALRLAID DADITWRPGLAFARPDALPIAW	411
LlpOVI	331	LDRKPNHL SFHGHIHCLGAQLGRMELEIALGALLRRMPGLRLAVPEADLQWSSQLAFSRPRTL PVAW	399
PdmW	329	VTRTDNPHLTF SHGTHYCAGASLARLELEVVLSTLIRRLPDLRLAIPATDLRRNDGNLLESFREIPITWG-GRG	401
PikC	337	IRRDTAGHLAFHGHIHFCIGAPLARLEARI A VRALLERCPDLALDVSPGELVWYPNMIRGLKALPIRWRRGREAGRRTG	416

Figure 8. Amino acid sequence alignment. PdmJ sequence aligned with three other actinomycete CYP monooxygenases. The identical amino acid residues are shadowed in grey. Three conserved regions in CYP monooxygenases are boxed



The gene *pdmJ* was cloned from the genomic DNA of the pradimicin producing strain and ligated into pJET1.2 to yield pZJ41. After confirming the sequence of the PCR product, *pdmJ* was ligated into pACYCDuet-1 to yield the expression plasmid pKN36. The plasmid was transformed into *E. coli* BL21(DE3) and expressed at 28°C. SDS-PAGE analysis of the soluble and insoluble fractions of the cell lysate indicated that both fractions contained PdmJ. The protein was then purified from the soluble fraction by Ni-NTA chromatography to homogeneity. PdmJ was found in the eluent by buffer A containing 25 mM imidazole (Figure 10). SDS-PAGE analysis showed that the His<sub>6</sub>-tagged enzyme was pure and had the correct size (47 kDa). The yield of PdmJ was estimated to be 17.8 mg/L using bicinchoninic acid (BCA) protein assay. The purified PdmJ was subjected to spectroscopic analyses. An absolute spectrum of 5  $\mu$ M ferric PdmJ in 50 mM Tris-HCl buffer (pH 7.9) with 20% glycerol generated at room temperature showed Soret,  $\alpha$  and  $\beta$  bands at 417, 570, and 535 nm, respectively.

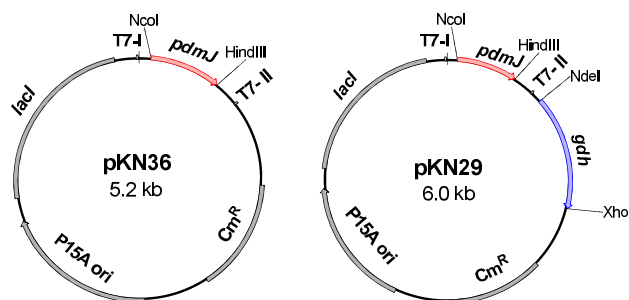


Figure 9. PdmJ and GDH expression plasmids. Maps of pKN36 (*pdmJ* in pACYCDuet-1 vector) and pKN29 (*pdmJ* and *gdh* in the pACYCDuet-1 vector).

Second-derivate analysis of the Soret peak indicated that about 90% of the iron was in the low spin state (Figure 11A). The CO-difference spectra<sup>20</sup> of PdmJ (Figure 11 B)

showed a peak at 450 nm immediately after the addition of dithionite to the enzyme saturated with CO, indicating that PdmJ is a CYP enzyme. However, the P450 form is unstable and rapidly changes to the P420 form, as revealed by the formation of the peak at 420 nm which increased after each reading (1-minute intervals) while the peak at 450 nm diminished.

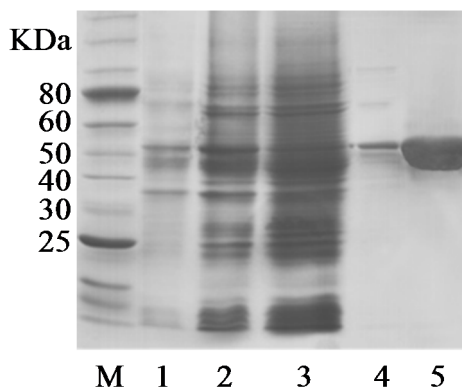


Figure 10. SDS-PAGE analysis of expression and purification of PdmJ. M: protein ladder; 1: insoluble protein; 2: soluble protein; 3: flow through; 4: elution with buffer A. 5: elution with buffer A containing 25 mM imidazole.

With the purified PdmJ in hand, we next tested its activity through *in vitro* reactions. In our previous *in vivo* study, heterologous expression of PdmJ with the enzymes (PdmABCDGHKL) that form **4** in *S. coelicolor* CH999 yielded the hydroxylated product **5**.<sup>14</sup> This indicated that **4** is a substrate of PdmJ. Accordingly, we isolated **4** from *S. coelicolor* CH999/pJX120 (Figure 12A, trace i) and used it as the substrate in our *in vitro* reactions. LC-MS analysis of the reaction mixture showed that PdmJ had converted **4** into a more polar product (ca. 47.8% yield) at 19.6 minutes (Figure 12 A, trace ii). This product was identified as **5** by a comparison of the UV/Vis spectrum (Figure 37), molecular weight

(448 Da, Figure 36), and retention time with those of the authentic sample, clearly confirming that PdmJ is the C-5 hydroxylase, which is in agreement with the *in vivo* result previously reported.<sup>14</sup>

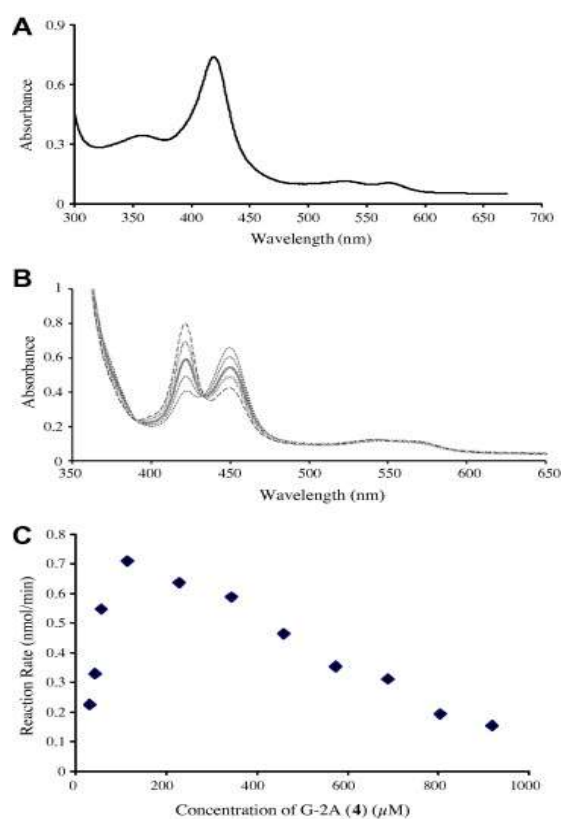


Figure 11. Spectroscopic and enzymatic analyses of PdmJ. (A) Absolute spectrum of ferric PdmJ. (B) CO-difference spectra of PdmJ. (C) Substrate inhibition of PdmJ-catalyzed C-5 hydroxylation of **4**.

To study the kinetics of PdmJ, a wide range of concentrations of **4** (32-920 μM) were tested in the reactions. As shown in Figure 11C, when the substrate was present at low concentrations, the reaction rate increased with the concentration. The reaction rate reached the highest level, 0.7 nmol/min, when the concentration of **4** was 115 μM. When more substrate was provided, the reaction rate declined. A non-Michaelis-Menten kinetics

curve was obtained from the experimental data (Figure 11 C), likely due to the substrate inhibition at the higher concentrations. It is known that **1-3** are the major secondary metabolites of *A. hibisca* P157-2. The isolation yields of **1-3** in the production medium were 290.9, 26.8 and 18.8 mg/l, respectively. It is proposed that the enzymes in the pradimicin biosynthetic pathway are controlled in such a collaborative way that the intermediates can be efficiently utilized by the immediate downstream enzymes. Consequently, the physiological concentration of the biosynthetic intermediates including **4** in *A. hibisca* P157-2 is low and substrate inhibition of PdmJ in this pradimicin producing strain does not occur. In fact, it is normal in metabolism that the concentrations of intracellular intermediates are much lower than the metabolic fluxes. This explains the high yields of **1-3** in *A. hibisca* P157-2 despite the substrate inhibition of PdmJ.

To examine the substrate specificity of PdmJ, JX137a (**6**) (Table 2), a D-Ala containing analogue of **4**, was isolated from *S. coelicolor* CH999/pJX137 and used for the hydroxylation assay. **6** is highly similar to **4** except that it contains a D-Ala moiety (Figure 36 and Figure 37). However, no obvious formation of a hydroxylated product was detected (Figure 12, trace iv), which indicated that PdmJ has strict substrate specificity. Our previous study has shown that **6** is the product of coexpression of PdmN with PdmABCDGHLK in *S. coelicolor* CH999.<sup>14</sup> Since **6** is not an appropriate substrate of PdmJ, it is likely that the amino acid ligation catalyzed by PdmN is a tailoring step that occurs after C-5 hydroxylation. Given their similar structures, we also tested whether **6** can interfere with the hydroxylation of **4** by competing for the substrate binding site of PdmJ. In the presence of **6** at 200  $\mu$ M and 400  $\mu$ M, the conversion rates of **4** to **5** were reduced by

about 40% and 80%, respectively. This indicated that although **6** is not a substrate of PdmJ, it can still bind to the enzyme as a competitive inhibitor.

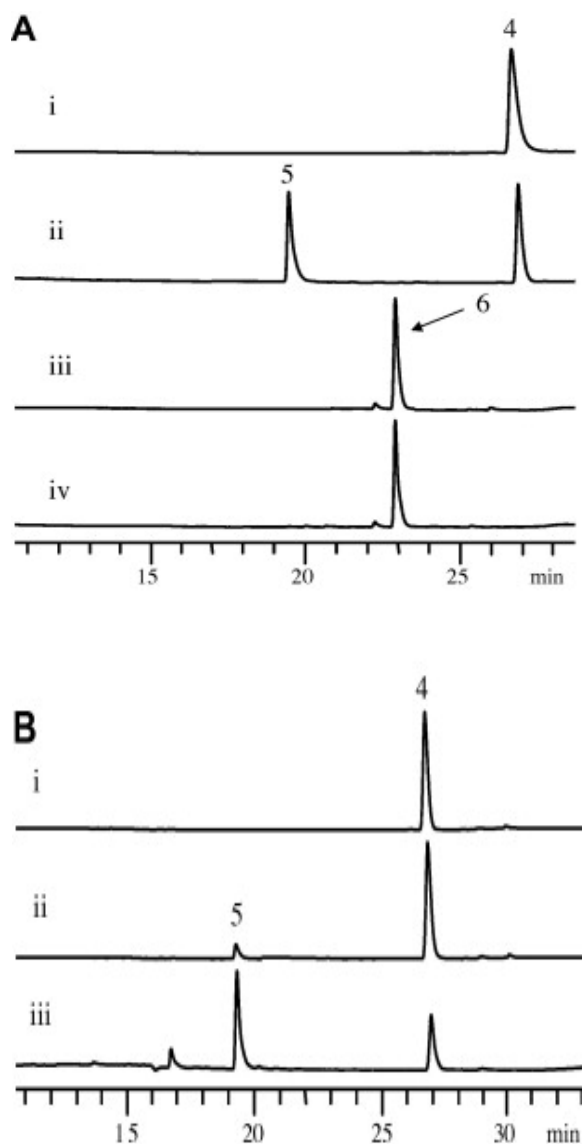


Figure 12. HPLC analysis of C-5 hydroxylation by PdmJ at 460 nm. (A) *In vitro* reaction of PdmJ with **4** and **6**. (i) Standard sample of **4**. (ii) The reaction mixture of PdmJ with **4**. (iii) Standard sample of **6**. (iv) The reaction mixture of PdmJ with **6**. (B) *In vivo* bioconversion of **4** to **5** by engineered *E. coli*. (i) *E. coli* BL21(DE3)/ pACYCDuet-1; (ii) *E. coli* BL21(DE3)/pKN36; (iii) *E. coli* BL21(DE3)/pKN29 with 5 g/L glucose.

Although lacking the flavoprotein NADPH-CYP reductase that ubiquitously exists in eukaryotic cells, *E. coli* has been found capable of supporting the activities of heterologous CYPs.<sup>21</sup> We next attempted to feed **4** to *E. coli* BL21(DE3)/pKN36 for its bioconversion to **5**. The substrate was added to the fermentation broth 3 h after IPTG induction and then maintained at 28°C for an additional 30 h. The culture was centrifuged and LC-MS analysis of the methanol extract of the sedimented cells showed that **4** was converted into **5** (ca. 7.3% yield) by the engineered strain (Figure 12 B, trace ii). In contrast, no product was found in the ethyl acetate extract of the supernatant fraction recovered after centrifugation (data not shown). No bioconversion was observed by the plasmid control strain *E. coli* BL21(DE3)/pACYCDuet-1 that lacks *pdmJ* (Figure 12 B, trace i).

Glucose 1-dehydrogenase (GDH) oxidizes D-glucose into D-glucono- $\delta$ -lactone in the presence of cofactor NAD<sup>+</sup> or NADP<sup>+</sup> to regenerate NAD(P)H. Coexpression of GDH with CYP enzymes significantly improves the *in vivo* efficiency of several CYP enzymes.<sup>22, 23</sup> We thus tested whether coexpression of GDH and PdmJ would improve the rate of bioconversion of **4** to **5**. Both *gdh* (from *Bacillus subtilis*) and *pdmJ* were cloned into pACYCDuet-1 to yield pKN29 that harbors the genes in two different multiple cloning sites and under the control of separate T7 promoters (Figure 9). As shown in Figure 12 B (trace iii), coexpression did show a significant improvement of the bioconversion (ca. 67.4 % yield) compared to *E. coli* BL21(DE3)/pKN36 when glucose was supplemented at 5 g/L in the fermentation broth, which is likely attributed to the efficient regeneration of NADPH. The yield may be further improved by coexpressing a NADPH-CYP reductase with PdmJ and GDH.

## 2.5 Conclusions

Pradimicins represent a novel family of medicinally important natural products because of their significant antifungal and antiviral activities. Understanding of the biosynthetic mechanism of these pentangular polyphenols will advance understanding of polyketide biosynthesis and provide a basis for engineering biosynthesis of “unnatural” natural products for new drug discovery.

We have previously investigated the early biosynthetic steps using *in vivo* combinatorial biosynthesis approach. Given the importance of the 5-OH in pradimicin biosynthesis and the inefficient synthesis of **5** in the *Streptomyces* system, we reconstituted PdmJ in *E. coli* BL21(DE3) in this study and obtained the pure enzyme for biochemical characterization. Our *in vitro* studies revealed that PdmJ can take **4** as the substrate and efficiently introduce a hydroxyl group to the molecule at C-5 to form **5**. This hydroxyl group serves as a site for the subsequent glycosylations. The enzyme has narrow substrate specificity, as revealed by the fact that it failed to hydroxylate the highly similar compound **6**. Interestingly, the activity of this enzyme can be inhibited by **4** (substrate inhibition) and **6** (competitive inhibition). Future structural analysis of the enzyme may reveal valuable information about the substrate binding and catalytic sites of PdmJ. Although substrate inhibition of CYP enzymes has been previously studied in drug metabolism,<sup>24, 25</sup> this is the first time that a CYP hydroxylase in a type II polyketide biosynthetic pathway was found to be inhibited by its substrate. *In vivo* bioconversion of **4** to **5** was also achieved at a high yield by feeding the substrate to the induced fermentation broth of the *E. coli* strain that expresses PdmJ and GDH.

Additionally, it should be noted that the bioconversion of **4** to **5** can be done in 2 days, rather than 8 days that is required for *S. coelicolor* CH999/pJX134 to produce **5**.<sup>14</sup> Thus, bioconversion by *E. coli* BL21(DE3)/pKN29 is useful for producing **5** as an important pradimicin biosynthetic intermediate, which can be used as a starting molecule for further chemical or enzymatic glycosylations to generate novel pradimicin analogues.

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## CHAPTER 3

### SYNERGISTIC ACTIONS OF TAILORING ENZYMES IN PRADIMICIN BIOSYNTHESIS<sup>2</sup>

#### 3.1 Abstract

Three key tailoring enzymes in pradimicin biosynthesis: PdmJ, PdmW, and PdmN, were investigated for their synergistic actions. PdmW was characterized as the C-6 hydroxylase by structural characterization of the corresponding product, 6-hydroxy-G-2A. The efficiencies of the C-5 and C-6 hydroxylations, catalyzed respectively by PdmJ and PdmW, were low when they were expressed individually with the early biosynthetic enzymes that form G-2A. When these two cytochrome P450 enzymes were co-expressed, a dihydroxylated product, 5,6-dihydroxy-G-2A, was efficiently produced, indicating that these two enzymes work synergistically in pradimicin biosynthesis. Heterologously expressed PdmN in *Streptomyces coelicolor* CH999 converted G-2A to JX137a by ligating a unit of D-alanine to the carboxyl group. PdmN has relaxed substrate specificity toward both amino acid donors and acceptors. Through combinatorial biosynthesis, a series of new pradimicin analogues were produced.<sup>1</sup>

#### 3.2 Introduction

The pradimicin family of natural products was first isolated in 1988 from the soil bacterium *A. hibisca* P157-2 (ATCC 53557).<sup>2</sup> Pradimicin A (**1**) is a major product from

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<sup>2</sup> Co-authors: Zhang, S.; Morgan, W.; Anderson, T.; Takemoto, J. Y.; and Zhan, J.

this strain. Its structure features a benzo[ $\alpha$ ]naphthacenequinone core, a D-alanine moiety, and a disaccharide moiety consisting of 4',6'-dideoxy-4'-methylamino-D-galactose and D-xylose.<sup>3</sup> *In vitro* and *in vivo* studies indicated that **1** is a promising lead compound for development of antifungal and antiviral drugs.<sup>4, 5, 6</sup> It has shown significant antifungal activity against many clinically important pathogens, such as *Candida*, *Aspergillus*, *Microsporum*, *Penicillium*, and *Sporothrix*.<sup>3, 5</sup> It is also a virus entry inhibitor and possesses selective activities against viruses such as the human immunodeficiency virus (HIV).<sup>7</sup> Recent studies on the mechanism of action of **1** highlighted its lectin-like property in a  $\text{Ca}^{2+}$ -dependent manner.<sup>8, 9</sup> The carboxyl group of D-alanine at C-18 and the CDE rings of **1** form the cavity that interacts with the D-mannopyranoside found in the surface envelope of several species of yeasts and in the glycoprotein gp120 of HIV.<sup>10</sup> This unique mechanism of action could help meet the challenges of multidrug-resistant yeast and provide the basis for new therapeutic approaches to prevent the entry of HIV into TCD4+ cells.<sup>7, 11</sup>

As the only family of natural carbohydrate-binding antibiotics with nonpeptidic skeletons (lectin mimics), pradimicins are a promising group of drug leads. To develop effective anti-infective drugs, BMY-28864 (**7**) was previously semi-synthesized, with improved water solubility ( $>40 \text{ g L}^{-1}$  at pH 2) and increased bioactivities over **1**<sup>12</sup> and its analogue, BMS-181184. However, BMY-28864 was found to increase hepatic transaminase level in healthy humans, leading to discontinuation of the clinical trial of this compound.<sup>13</sup> New analogues of **1** with improved biological activities and safety profiles are thus needed for new drug development.

Compound **1** belongs to a large family of natural products called polyketides. It is assembled via a type II polyketide biosynthetic pathway. Type II polyketide biosynthetic pathways consist of a series of discrete enzymes that work collaboratively to synthesize diverse molecules, such as the broad-spectrum antibiotic oxytetracycline.<sup>14</sup> Unlike modular type I polyketide synthases (PKSs) and simple type III PKSs, type II PKSs form multi-enzyme complexes to act as an efficient assembly line. For example, the KS and CLF form a heterodimer to precisely control chain elongation. Together with a dissociated ACP, they synthesize a linear poly- $\beta$ -ketone chain of the desired length and, thus, are called minimal PKSs. The nascent polyketide chain is then cyclized and modified by tailoring enzymes to yield final products.

Combinatorial biosynthesis is an attractive approach to generating new molecules. In order to rationally engineer the biosynthesis of new pradimicin analogues, it is important to functionally characterize the involved enzymes, understand their catalytic properties, and gain insights into their synergistic actions in the biosynthetic process. The biosynthetic gene cluster of **1** has been previously reported.<sup>15</sup> Two ABC transporter genes, *pdmR1* and *pdmR2*, were found to act as resistance genes. While overexpression of these genes in *A. hibisca* P157-2 led to elevated production of **1**, their introduction into pradimicin-sensitive microorganisms, such as *Streptomyces venezuelae*, increased their tolerance to **1**.<sup>16</sup>

In our previous work, we functionally characterized and identified the early steps in the biosynthetic pathway of **1**. The minimal PKS, which consists of PdmA (KS), PdmB (CLF), and PdmC (ACP), assembles the nascent dodecaketide backbone from 12 units of malonyl-CoA. This 24-carbon poly- $\beta$ -ketone chain is then processed by immediate

tailoring enzymes including PdmD, PdmK, PdmL, PdmH, and PdmG to give rise to an important benzo[ $\alpha$ ]naphthacenequinone intermediate, G-2A (**4**) (Table 2), with an average yield of  $48.8 \pm 3.2 \text{ mg L}^{-1}$  in the heterologous host *Streptomyces coelicolor* CH999.<sup>17</sup> PdmN was characterized as an amino acid ligase that ligates a molecule of D-alanine to the carboxyl group at C-16.<sup>14</sup> A new pradimicin analogue JX137a (**6**) was synthesized at  $31.9 \pm 3.1 \text{ mg L}^{-1}$  when PdmN was co-expressed with the enzymes that form **4**. This enzyme could also accept D-serine as the donor to yield another derivative, JX137s. PdmJ was characterized both *in vivo* and *in vitro* as a cytochrome P450 (CYP450) enzyme that specifically introduces a hydroxy group at C-5. Hydroxylation of **4** by PdmJ yielded JX134 (**5**).<sup>14, 18</sup> This hydroxy group served as a site for subsequent glycosylations. Given the essential roles of the D-alanine and sugar moieties in the biological activities of **1**, it is important to understand the catalytic properties of these tailoring enzymes and how they work together in pradimicin biosynthesis.

In this work, the three key tailoring enzymes, including PdmW, PdmJ, and PdmN, were further investigated. PdmW was determined to be the C-6 hydroxylase that acts synergistically with the C-5 hydroxylase, PdmJ. PdmN was discovered to possess broad acceptor substrate specificity, and a series of new pradimicin analogues was generated through combinatorial biosynthesis.

### 3.3 Materials and Methods

#### 3.3.1 General

1D and 2D NMR spectra were recorded on a JEOL instrument (300 MHz). ESI-MS were acquired on an Agilent 6130 LC-MS. The extracts were analyzed on an Agilent

1200 HPLC instrument by using an Agilent ZORBAX SB-C18 column (5  $\mu$ m, 4.6 mm  $\times$  150 mm), eluted with a gradient of CH<sub>3</sub>CN/water (25–90%) with 0.1% formic acid over 25 minutes at a flow rate of 1 mL min<sup>-1</sup>, followed by 100% CH<sub>3</sub>CN for 5 min. Compounds were purified on the same HPLC instrument. The yields of the products from three different colonies of the same strain were analyzed by HPLC and determined by using the standard curves of the purified compounds.

### 3.3.2 Bacterial strain and vectors

*E. coli* XL-1 Blue (Stratagene) was used for routine subcloning. *S. coelicolor* CH999 was used for the expression of *pdm* biosynthetic genes. *A. hibisca* P157-2 was obtained from the American Type Culture Collection (ATCC). The cloning vectors used in this study were pCR-Blunt (Life Technologies) and pJET1.2 (Fermentas). The pRM5 *E. coli*/*Streptomyces* shuttle vector was used to construct the expression plasmids.

### 3.3.3 Growth media and conditions

*E. coli* strains were routinely grown in lysogeny broth (LB) at 37 °C. For selection of correct transformants, ampicillin (100 mg mL<sup>-1</sup>) or kanamycin (50 mg mL<sup>-1</sup>) were added as supplements. *S. coelicolor* CH999 was used as the heterologous host for expression of the pradimicin biosynthetic enzymes. The expression plasmids were introduced into *S. coelicolor* CH999 through polyethylene glycol (PEG)-mediated protoplast transformation. The transformants were selected on R5 agar supplemented with 50 mgmL<sup>-1</sup> thiostrepton. All of the engineered strains of *S. coelicolor* CH999 were cultivated on R5 agar with thiostrepton at 28 °C for production of the polyketide compounds.

### 3.3.4 Construction of plasmids

The genes used in this study were amplified by PCR. Specific forward and reverse primers were designed, based on the reported sequences, to contain compatible restriction enzymes such as XbaI, SpeI, and NheI. The PCR products were then ligated into a cloning vector for DNA sequencing. Each combination of genes was constructed by the subsequent addition of individual genes into a cloning vector and restriction enzyme digestions confirmed the correct ligation. Additionally, each group of genes was ligated into a pRM5-derived vector. The gene cassette (*pdmCGHKLJ*) was excised from pKN31 by using XbaI and SpeI and ligated into the pRM5-derived vector pJX111 (*pdmABD*) between the same sites to yield pKN35. The *pdmN* gene was excised from pJX97 by using XbaI and SpeI and ligated into the XbaI treated pKN35 to yield pKN89. Similarly, the same insert was ligated into XbaI-digested pJX120 to afford pKN93. The *pdmW* gene was excised from pJX151 by using XbaI and NheI and ligated into XbaI-digested pKN93 to yield pKN102. The same insert was ligated into XbaI-digested pJX120 to yield pKN92. When *pdmW* was ligated into XbaI-digested pKN35, pKN90 was constructed. pKN87 was generated by ligating *pdmWCGHIKLNJ* into pJX111 between XbaI and SpeI. The *pdmN* gene was excised from the pKN39 plasmid by using EcoRI and PacI and ligated into the pRM5 vector between the same sites to yield the plasmid pKN50. All of these plasmids were introduced into *S. coelicolor* CH999 through PEG-mediated protoplast transformation for product analysis or biotransformation.

### 3.3.5 In vivo D-alanylation

*S. coelicolor* CH999/pKN50 was cultivated in R5 broth containing 50 mg mL<sup>-1</sup> thiostrepton at 28 °C for 4 days in a rotary shaker. This seed culture (100 mL) was



transferred to 10 mL of fresh R5 broth with 50 mg mL<sup>-1</sup> thiostrepton and cultured for 4 days before the addition of 0.5 mg of **4**, **5**, **8**, or **9** dissolved in pure DMSO. After 10 days of biotransformation, the cultures were centrifuged at 3220 g for 10 min. The pelleted cells were extracted with methanol. This MeOH extract was combined with the supernatant and dried in vacuo. The samples were subjected to LC-MS analysis.

### 3.3.6 Extraction and isolation of compounds

The engineered *S. coelicolor* CH999 strains were grown on R5 agar with 50 mg mL<sup>-1</sup> thiostrepton at 28 °C for 8–10 days. Products were extracted from the cultures by using a mixture of ethyl acetate, MeOH, and acetic acid (89:10:1). The extracts were then dried under reduced pressure. The extract of 6 L of *S. coelicolor* CH999/pKN92 was subjected to Diaion HP-20 resin column chromatography, eluted with 0, 25, 50, 75, and 100% aqueous MeOH (v/v) to yield five fractions. HPLC analysis revealed that the 75% fraction contained the target peak. This fraction was further separated by reversed-phase HPLC (Agilent Zorbax SB-C18, 5 µm, 21.2 mm × 150 mm) and eluted with 55% aqueous CH<sub>3</sub>CN containing 0.1% formic acid, at a flow rate of 1 mL min<sup>-1</sup>, to yield 6.0 mg of **8**. The extract of 2 L of *S. coelicolor* CH999/pKN90 was subjected to Diaion HP-20 resin column chromatography as described above. The 50% fraction that contained the target peak was further separated by reversed-phase HPLC (SOPELCO C18, 5 µm, 10 mm × 150 mm), eluted with a linear gradient of 15–95% aqueous CH<sub>3</sub>CN containing 0.1% formic acid, at a flow rate of 1 mL min<sup>-1</sup>, over 16 minutes to yield 8.5 mg of **9**. The extract of 6 L of *S. coelicolor* CH999/pKN102 was subjected to Diaion HP-20 resin column chromatography as described above. The 75% fraction was further separated by reversed-phase HPLC (Agilent Zorbax SB-C18, 5 µm, 21.2 mm × 150 mm), eluted with 37%

aqueous CH<sub>3</sub>CN containing 0.1% formic acid, at a flow rate of 1 mL min<sup>-1</sup>, to yield 1.0 mg of **10**. <sup>1</sup>H NMR data of **10** in [*d*<sub>6</sub>] DMSO: δ=9.02 (s, 1H; H-14), 7.12 (brs, 1H; H-12), 6.61 (s, 1H; H-4), 6.45 (brs, 1H; H-10), 5.22 (brs, 1H; H-6), 4.20 (m, 1H; H-17), 2.97 (m, 1H; H-5a), 2.76 (m, 1H; H-5b), 2.23 (s, 3H; H-15), 1.27 ppm (d, *J*= 7.2 Hz, 3H; H-19). The extract of 2 L of *S. coelicolor* CH999/pKN87 was subjected to Diaion HP-20 resin column chromatography as described above. The 50% fraction was found to contain the target peak. This fraction was dissolved in DMSO after solvent evaporation and then subjected to a HW-40F chromatographic column (30 mm x 100 mm), eluted with a gradient of MeOH/water, to remove the non-pradimicin compounds. The 50% MeOH/water elute was further separated by HPLC (ZORBAX SB-C18, 5 μm, 4.6 mm × 150 mm) and eluted with 23% aqueous CH<sub>3</sub>CN containing 0.1% formic acid, at a flow rate of 1 mL min<sup>-1</sup>, to yield 8.1 mg of **11**.

### 3.3.7 Determination of the absolute configuration of **5** and **8**

In order to determine the absolute configurations of **5** at C-5 and **8** at C-6, these compounds were reacted with (S)- and (R)-MTPA chloride separately to afford the corresponding (R)- and (S)-MTPA esters **5R**, **5S** and **8R**, **8S**. Their <sup>1</sup>H, <sup>19</sup>F, and <sup>1</sup>H, <sup>1</sup>H COSY NMR spectra were recorded in [D<sub>5</sub>]pyridine after reacting for 24 h at room temperature.

### 3.3.8 Antifungal assays of **4-11**

The antifungal activities of compounds **4-11** were tested against *C. albicans* ATCC MYA-2876. The inoculum was prepared by cultivating a single colony of *C. albicans* in 5 mL of potato dextrose broth (PDB) supplemented with 200 mM CaCl<sub>2</sub> at 35 °C for 24 h. The inoculum was diluted with PDB and titrated with a hemocytometer to reach a final

inoculum size of  $2 \times 10^6$  CFU mL<sup>-1</sup>. Stock solutions of each test compound were prepared in 50% aqueous DMSO with a concentration of 5 mg mL<sup>-1</sup>. The test concentrations ranged from 0.9 to 500 mg mL<sup>-1</sup>. Clotrimazole was dissolved in pure DMSO and used as a positive control.

### 3.4 Results and Discussions

#### 3.4.1 Characterization of PdmW as the C-6 hydroxylase

To identify the function of PdmW, we constructed plasmid pKN92 (Table 6), which consists of *pdmW* and the G-2A-forming genes, by using an *E. coli*/*Streptomyces* shuttle vector pRM5.<sup>19</sup> This vector contains a ColE1 replicon for replication in *E. coli* and a SCP2 replicon for *Streptomyces*. For comparison, a similar plasmid pKN35 was constructed containing the same set of genes except that *pdmW* was replaced by *pdmJ* (Table 6). These two plasmids were introduced into *S. coelicolor* CH999 by protoplast transformation, and correct transformants were grown on R5 agar supplemented with thiostrepton for product analysis.

Table 6. Plasmids constructed in this study and corresponding polyketide products.

Plasmid	Genes	Products	Ref.
pJX120	<i>pdmABCDGEHIKL</i>	4	<sup>17</sup>
pKN35	<i>pdmABCDGEHIKLJ</i>	4, 5	This work
pKN92	<i>pdmABCDGEHIKLW</i>	4, 8	This work
pKN93	<i>pdmABCDGEHIKLN</i>	4, 6	This work
pKN89	<i>pdmABCDGEHIKLNJ</i>	4, 6	This work
pKN90	<i>pdmABCDGEHIKLJW</i>	4, 5, 8, 9	This work
pKN102	<i>pdmABCDGEHIKLNW</i>	4, 6, 8, 10	This work
pKN87	<i>pdmABCDGEHIKLJNW</i>	4, 6, 5, 8, 9, 11	This work
pKN50	<i>pdmN</i>	n.a.	This work

The products were extracted with ethyl acetate/methanol/acetic acid (89:10:1) and analyzed by LC-MS. As expected, *S. coelicolor* CH999/pKN35 (Figure 13, trace i) produced **4** as the major product and the hydroxylated analogue **5** as a minor metabolite ( $1.7 \pm 0.2 \text{ mg L}^{-1}$ ), which is consistent with our previous discovery.<sup>14</sup> In contrast, *S. coelicolor* CH999/ pKN92 (Figure 13, trace ii) produced a different minor compound **8** ( $1.4 \pm 0.4 \text{ mg L}^{-1}$ ) in addition to **4**. This compound has a UV/Vis absorption spectrum similar to that of **4** (Figure 37), indicating that they share a similar structure. ESI-MS revealed that its molecular weight is 448 (Figure 36), 16 amu larger than **4**, suggesting that it is a hydroxylated product of **4**. As **5** and **8** have different retention times, it was proposed that the latter has a hydroxy group at a position other than C-5. We previously detected a similar compound produced by *S. coelicolor* CH999/pJX152 possessing the same set of genes, but it was not structurally characterized, due to the lack of sufficient amounts of pure compound.<sup>14</sup> In this work, we scaled up the culture to 6 L and purified 6.0 mg of **8** for NMR analysis (Figure 38).

The <sup>13</sup>C NMR spectrum of **8** revealed that it has 24 carbons, confirming that it is a dodecaketide synthesized by the pradimicin biosynthetic enzymes (Figure 39). Compared to **4**, which has a CH<sub>2</sub> group at both C-5 and C-6, **8** has a single CH<sub>2</sub> group but contains a new oxygenated CH group. This suggested that the hydroxy group was introduced at C-5 or C-6. Because **5** was already identified as 5-hydroxy-G-2 A, **8** was deduced to be a C-6-hydroxylated derivative of **4**. Further 2D NMR analysis of **8** (Figure 14) revealed <sup>1</sup>H–<sup>1</sup>H COSY correlation of the oxygenated CH signal to 5-CH<sub>2</sub> (Figure 40) and its HMBC correlation to C-4a (Figure 41), thus confirming that **8** is 6-hydroxy-G-2A, which was named KN92 (Table 2). The proton and carbon signals were assigned and are presented in

Table 7 and Table 8, respectively. We then prepared the MTPA esters of **8** to determine the configuration of C-6. As shown in Figure 14, the  $\Delta\delta$  values revealed that C-6 has *R* configuration. Full structural characterization of **8** confirmed that PdmW was the dedicated C-6 hydroxylase in pradimicin biosynthesis. Using the same method, we also determined the absolute configuration at C-5 of **5** to be *R* (Figure 15), which was not determined previously. We also recorded the  $^{19}\text{F}$  NMR data of these Mosher esters, which supported the determination of the absolute configurations of **5** and **8** (Figure 35).<sup>20</sup> Therefore, this work allowed the characterization of PdmW as the C-6 hydroxylase and the *R* configuration at C-5 in **5** and C-6 in **8**, which are consistent with the reported absolute configurations at the same positions in final product **1**.

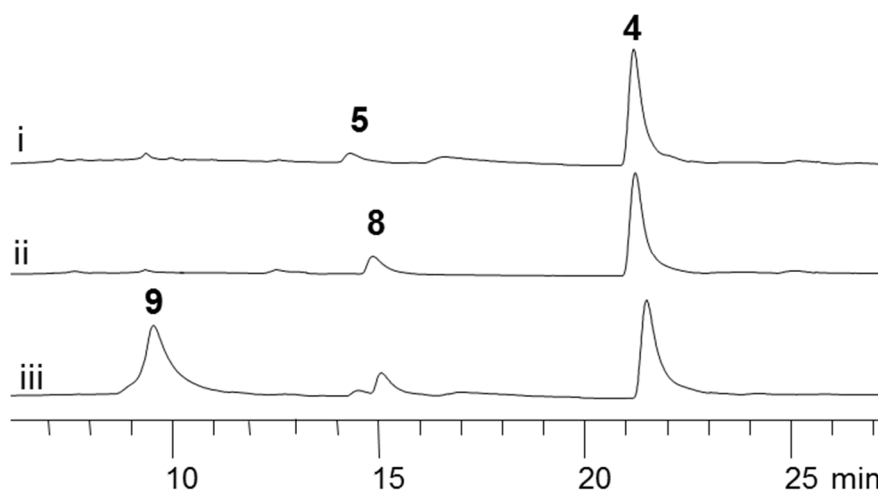


Figure 13. Synergistic actions of two CYP hydroxylases in pradimicin biosynthesis. (i) HPLC trace of *S. coelicolor* CH999/pKN35; (ii) HPLC trace of *S. coelicolor* CH999/pKN92; (iii) HPLC trace of *S. coelicolor* CH999/pKN90. These extracts were analyzed at 460 nm.

Table 7. The  $^1\text{H}$  NMR data (300MHz) of **8**, **9** and **11** (in DMSO- $d_6$ ,  $\delta$  in ppm,  $J$  in Hz).

Position	<b>8</b>	<b>9</b>	<b>11</b>
4	6.64 (1H, s)	6.89 (1H, s)	6.83 (1H, s)
5	2.83-2.98 (2H, m)	4.50 (1H, d, $J=3.0$ )	4.49 (1H, d, $J=3.1$ )
6	5.16 (1H, s)	5.09 (1H, d, $J=2.1$ )	5.09 (1H, d, $J=2.8$ )
10	6.57 (1H, s)	6.64 (1H, d, $J=2.4$ )	6.64 (1H, d, $J=1.7$ )
12	7.12 (1H, s)	7.19 (1H, d, $J=2.4$ )	7.19 (1H, d, $J=2.0$ )
14	8.82 (1H, s)	8.93 (1H, s)	8.87 (1H, s)
15	2.47 (1H, s)	2.57 (1H, s)	2.36 (1H, s)
17			4.47 (1H, m)
19			1.39 (1H, d, $J=7.2$ )
7-OH	12.47 (1H, s)	12.54 (1H, s)	12.54 (1H, s)
9-OH	12.03 (1H, s)	12.13 (1H, s)	12.14 (1H, s)
11-OH	11.46 (1H, brs)	11.42 (1H, s)	11.62 (1H, brs)
16-NH			8.86 (1H, d, $J=5.9$ )

Table 8. The  $^{13}\text{C}$  NMR data (75MHz) of **8**, **9** and **11** (in DMSO- $d_6$ ,  $\delta$  in ppm).

Position	<b>8</b>	<b>9</b>	<b>11</b>	Position	<b>8</b>	<b>9</b>	<b>11</b>
1	162.3	160.8	153.1	11	165.7	165.9	165.8
2	113.1	113.8	125.3	12	108.9	109.0	108.9
3	143.1	144.3	137.8	12a	135.3	135.3	135.4
4	123.4	119.6	124.3	13	181.4	181.9	181.7
4a	141.9	143.3	140.1[a]	13a	131.3[a]	131.5	131.6[a]
5	37.2	71.0	70.9	14	119.9	119.6	119.8
6	56.9	62.1	62.4	14a	140.0	139.2	140.4[a]
6a	131.5[a]	132.1	131.4[a]	14b	117.2	116.9	116.8
7	158.5	160.2	160.0	15	23.7	23.3	19.4
7a	113.2	114.3	113.4	16	173.9	174.2	167.5
8	189.3	190.1	189.6	17			48.2
8a	109.1	109.1	109.2	18			174.7
9	164.5	164.5	164.5	19			16.5
10	107.9	108.3	107.9				

**[a] Assignment interchangeable.**

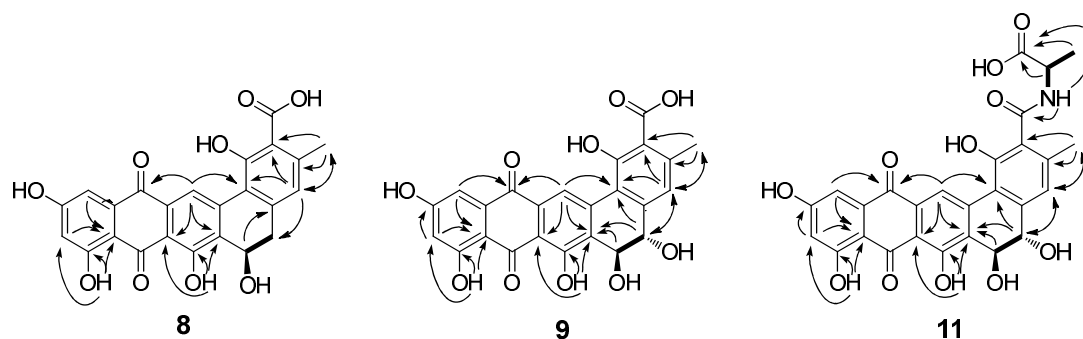


Figure 14. Key  $^1\text{H}$ - $^1\text{H}$  COSY and HMBC correlations for **8**, **9** and **11**.

### 3.4.2 Synergistic actions of two CYP hydroxylases in pradimicin biosynthesis

As described above, PdmJ and PdmW were characterized as CYP hydroxylases that introduce a hydroxy group to C-5 and C-6 of the pradimicin structure, respectively. However, the yields of **5** and **8** were low, as shown in Figure 13, traces i and ii. The major metabolite in both *S. coelicolor* CH999/pKN35 and *S. coelicolor* CH999/pKN92 was still : **4**, the benzo[a]naphthacenequinone intermediate formed by the biosynthetic genes *pdmABCDGHLK* in pJX120. Apparently, these CYP enzymes when expressed individually were not efficient in converting **4** into the corresponding monohydroxylated products. To test whether these two CYP hydroxylases work collaboratively, plasmid pKN90 that contains *pdmABCDGHLK* and both *pdmJ* and *pdmW* was constructed. Co-expression of these two hydroxylases in *S. coelicolor* CH999 resulted in the production of **5**, **8**, and a new product **9** ( $18.7 \pm 1.7 \text{ mg L}^{-1}$ ; Figure 13, trace iii). Compound **9** had a shorter retention time (9.6 min) than **5** and **8** on a reversed-phase C18 HPLC column, suggesting that it is more polar than the other two compounds. The UV/Vis absorptions of **9** (Figure 37) indicated that it is an analogue of **1**. ESI-MS spectra of **9** showed the ion peaks,

including  $[M-H]^-$  at  $m/z$  462.9 and  $[M+H]^+$  at  $m/z$  465.0, indicating that it has a molecular weight of 464 (Figure 36). This is 16 amu larger than **5** and **8**, indicating that **9** is a dihydroxylated product (Figure 36).

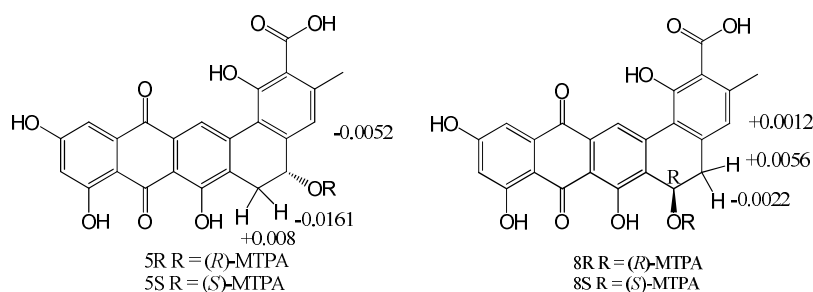


Figure 15. Determination of the absolute configurations of **5** and **8** using Mosher's method,  $\Delta\delta$  ( $\delta_S - \delta_R$  in ppm) values in pyridine- $d_5$ .

The  $^1\text{H}$  NMR spectrum of **9** exhibited 12 proton signals (Figure 42), including three hydroxy protons, four phenolic protons, two oxygenated CH protons, and a  $\text{CH}_3$  group (Table 7). The  $^{13}\text{C}$  NMR spectrum of **9** showed 24 carbons (Figure 43) including two carbonyl groups, one carboxyl group, 18 aromatic carbons, one methyl group, and two oxygenated CH groups (Table 8). The NMR data are very similar to those of **4**, **5**, and **8**. The major difference is that **9** does not have any  $\text{CH}_2$  groups, suggesting that both C-5 and C-6 are hydroxylated. Unlike the monohydroxylated compounds **5** and **8**, the  $^1\text{H}$  NMR signals for H-5 and H-6 were doublets at  $\delta = 4.50$  and 5.09 ppm, respectively. The  $^{13}\text{C}$  NMR and DEPT spectra showed that the chemical shifts of C-5 and C-6 were  $\delta$  71.0 (CH) and 62.1 ppm (CH), respectively.



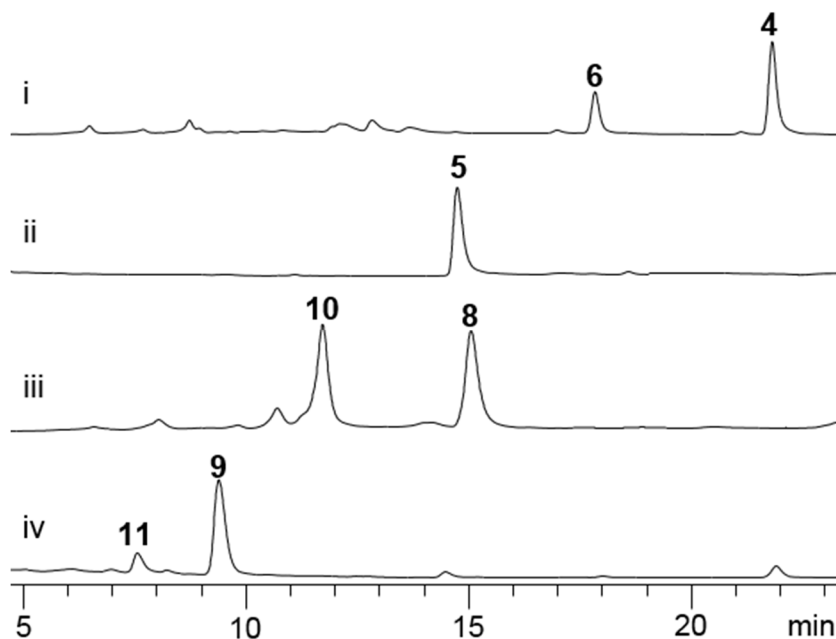


Figure 16. HPLC analysis of alanylation by PdmN. Biotransformation of **4** (i), **5** (ii), **8** (iii), and **9** (iv) by *S. coelicolor* CH999/pKN50 at 460 nm.

The  $^1\text{H}$ - $^1\text{H}$  COSY correlations between H-5 and H-6 (Figure 44), and the HMBC correlations (Figure 45) of H-4 to C-5 and H-6 to C-4a at  $\delta$  143.3, and H-5 to C-6a at 132.1 ppm, confirmed that C-5 and C-6 were hydroxylated (Figure 14). This was also supported by the  $^1\text{H}$ - $^1\text{H}$  ROESY correlations shown in Figure 34. Thus, **9** was characterized as 5,6-dihydroxy-G-2A, which was named KN90 (Table 2), according to the name of the producing plasmid, pKN90. We previously functionally expressed PdmJ in *E. coli*, and the engineered strain converted **4** into the C-5-hydroxylated product **5** with low efficiency. The conversion rate was improved when glucose dehydrogenase was coexpressed to recycle the cofactors.<sup>18</sup>

Although both PdmJ and PdmW can catalyze the corresponding hydroxylations of **4**, the efficiencies of both individual reactions were low. However, these two CYP enzymes work collaboratively to efficiently generate a new pradimicin biosynthetic intermediate **9**. A similar synergistic phenomenon with cyclases PdmK and PdmL was found in the formation of the pentangular structure of pradimicins, which require the presence of PdmH, a monooxygenase that oxidizes ring B into the quinone form, to close rings C–E.<sup>17</sup> It is also known that KS and CLF in type II polyketide biosynthetic pathways form a heterodimer to promote chain length control. In the case of PdmJ and PdmW, either enzyme can work separately, but it is apparent that both enzymes work more efficiently when coexpressed. Therefore, it is proposed that these two CYPs form a complex to catalyze the dihydroxylation of the substrate. Our previous work on PdmJ revealed substrate inhibition of this enzyme in the *in vitro* reactions.<sup>18</sup> The synergistic actions of PdmJ and PdmW are likely due to the prevention of substrate inhibition in pradimicin biosynthesis.

### 3.4.3 Broad substrate specificity of PdmN

Previously, PdmN was identified as an amino acid ligase that can accept D-alanine or D-serine to give different pradimicin analogues.<sup>14</sup> The relaxed amino acid substrate specificity of PdmN makes it a useful biocatalyst to generate novel pradimicin analogues. Previous attempts to express PdmN in *E. coli* BL21(DE3) by using the expression vector pET28a failed because an insoluble form of the enzyme was generated,<sup>18</sup> and similar results were obtained with other expression vectors such as pACYCDuet-1 and with different *E. coli* expression conditions. In the current work, PdmN expression in *S. coelicolor* CH999 was attempted, as functional expression of PdmN and other biosynthetic enzymes was

successful to afford **6**.<sup>14</sup> Expression plasmid pKN50 was constructed by ligating pdmN (1863 bp) into the pRM5 vector (Table 6). The plasmid was introduced into *S. coelicolor* CH999, and the transformant was used to test alanylation of different substrates, including **4**, **5**, **8**, and **9**. To confirm that this strain had functional PdmN, exogenously fed **4** was shown to be converted into its alanylated derivative **6** by cells of *S. coelicolor* CH999/pKN50 (Figure 16, trace i).

#### 3.4.4 Combinatorial biosynthesis of new pradimicin analogues

We next made a series of plasmids to generate new pradimicin analogues through combinatorial biosynthesis, which was achieved by combining one, two or all of the three tailoring enzymes (PdmN, PdmJ and PdmW) with the early biosynthetic enzymes (Table 6). All genes are transcribed from a single promoter/regulator *actI/actII-ORF4*. Ligation of *pdmN* into pJX120 yielded pKN93 (Table 6), which was introduced into *S. coelicolor* CH999. *S. coelicolor* CH999/pJX120, which produces **4** as the major product, was used as the control (Figure 17, trace i). LC-MS analysis of the culture of *S. coelicolor* CH999/pKN93 in R5 agar showed that **6** was produced in a high yield ( $31.9 \pm 3.1 \text{ mg L}^{-1}$ ), with **4** as the minor product (Figure 17, trace ii). When PdmJ was introduced into the system (pKN89), however, the same products as produced by *S. coelicolor* CH999/pKN93 were obtained (Figure 17, trace iii). This result suggested that, although both PdmN and PdmJ can use **4** as the substrate for C-16 alanylation and C-5 hydroxylation, respectively, it is apparent that PdmN has higher affinity for the intermediate **4** than does PdmJ. Compound **6** was efficiently synthesized from **4** by PdmN. PdmJ cannot further hydroxylate **6** to yield the corresponding alanylated and C-5 hydroxylated product. Accordingly, **5** was not observed, due to the low efficiency of the C-5 hydroxylation. This

is consistent with the *in vitro* results from our previous work.<sup>18</sup> In contrast, when pKN102 harboring *pdmABCDGHLNW* was expressed in *S. coelicolor* CH999, several products, including **4**, **6**, **8**, and **10** ( $0.3 \pm 0.1$  mg L<sup>-1</sup>) were biosynthesized (Figure 17, trace iv). The UV/Vis spectrum of **10** suggested that it was a pradimicin analogue (Figure 37). ESI-MS of **10** showed the  $[M-H]^-$  peak at  $m/z$  518 (Figure 36), indicating that it has a molecular weight of 519, which is 71 amu larger than that of **8** (Figure 36). This suggested that **10** (Table 2) is the alanylated derivative of **8** and the same as the biotransformation product of **8** by PdmN (Figure 16, trace iii).

Although the <sup>13</sup>C NMR spectrum was not acquired, due to the lack of sufficient amounts of **10**, the presence of the oxygenated H-6, as well as H-17 and CH<sub>3</sub>-19 from the D-alanine moiety, was confirmed by the proton signals at  $\delta = 5.16$  (s, 1H), 4.20 (s, 1H), and 1.27 ppm (s, 3H), respectively, in the <sup>1</sup>H NMR of **10**. Accordingly, the structure of **10** was deduced to be 6-hydroxy-JX137a. Therefore, in addition to **4**, which was formed by PdmABCDGHL, **6** and **8** resulted from the individual tailoring actions catalyzed by PdmN and PdmJ, respectively, and **10** was generated by the actions of both enzymes. However, the overall efficiency was still low, and **4** was accumulated as the major metabolite. Apparently, coexpression of PdmJ or PdmW with PdmN and other early biosynthetic enzymes yielded no or small amounts of the expected hydroxylated and alanylated product ( $0.3 \pm 0.1$  mg L<sup>-1</sup> of **10**), suggesting that PdmN cannot efficiently work with either of the two CYP enzymes. As we found that PdmJ and PdmW work synergistically to generate the dihydroxylated product **9**, we hypothesized that collaboration of PdmN with both CYP enzymes might be required to generate a new pradimicin analogue.

To this end, we coexpressed these three enzymes with the G-2A-forming enzymes by constructing a new expression plasmid, pKN87 (Table 6). Expression of this plasmid in *S. coelicolor* CH999 led to the production of a series of products, including **4**, **6**, **8**, **9**, and **11**, with **11** being the most predominant product ( $32.4 \pm 2.2$  mg L<sup>-1</sup>).

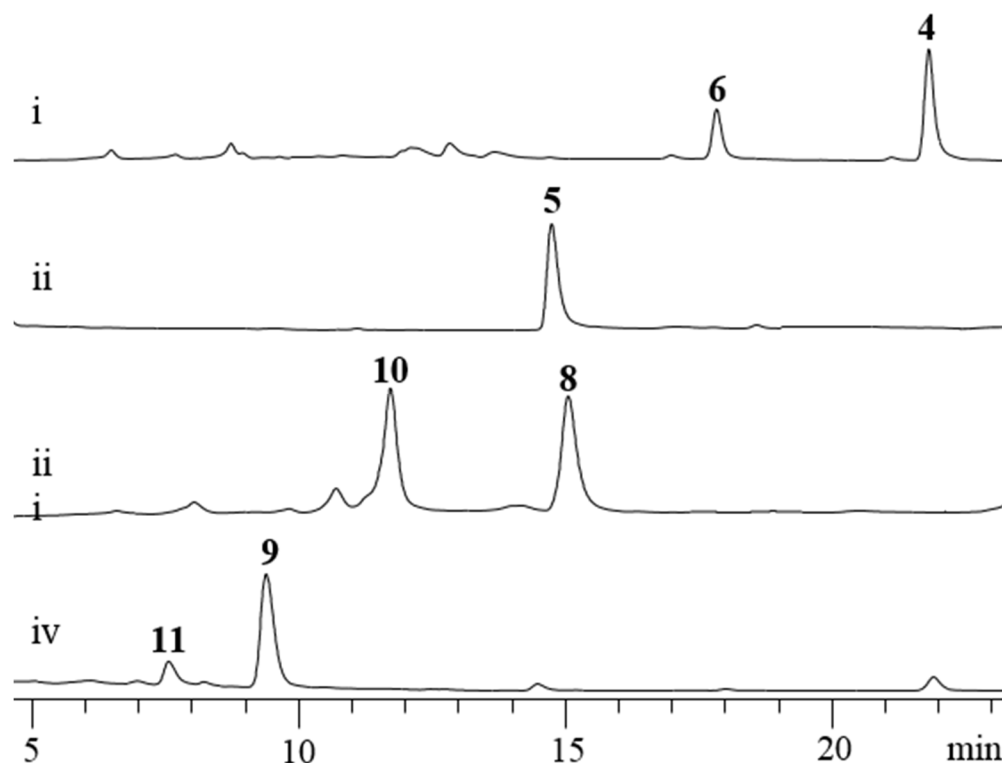


Figure 17. Combinatorial biosynthesis of new pradimicin analogues. (i) HPLC trace of *S. coelicolor* CH999/pJX120; (ii) HPLC trace of *S. coelicolor* CH999/pKN93; (iii) HPLC trace of *S. coelicolor* CH999/pKN89; (iv) HPLC trace of *S. coelicolor* CH999/pKN102; (v) HPLC trace of *S. coelicolor* CH999/pKN87. These extracts were analyzed at 460 nm.

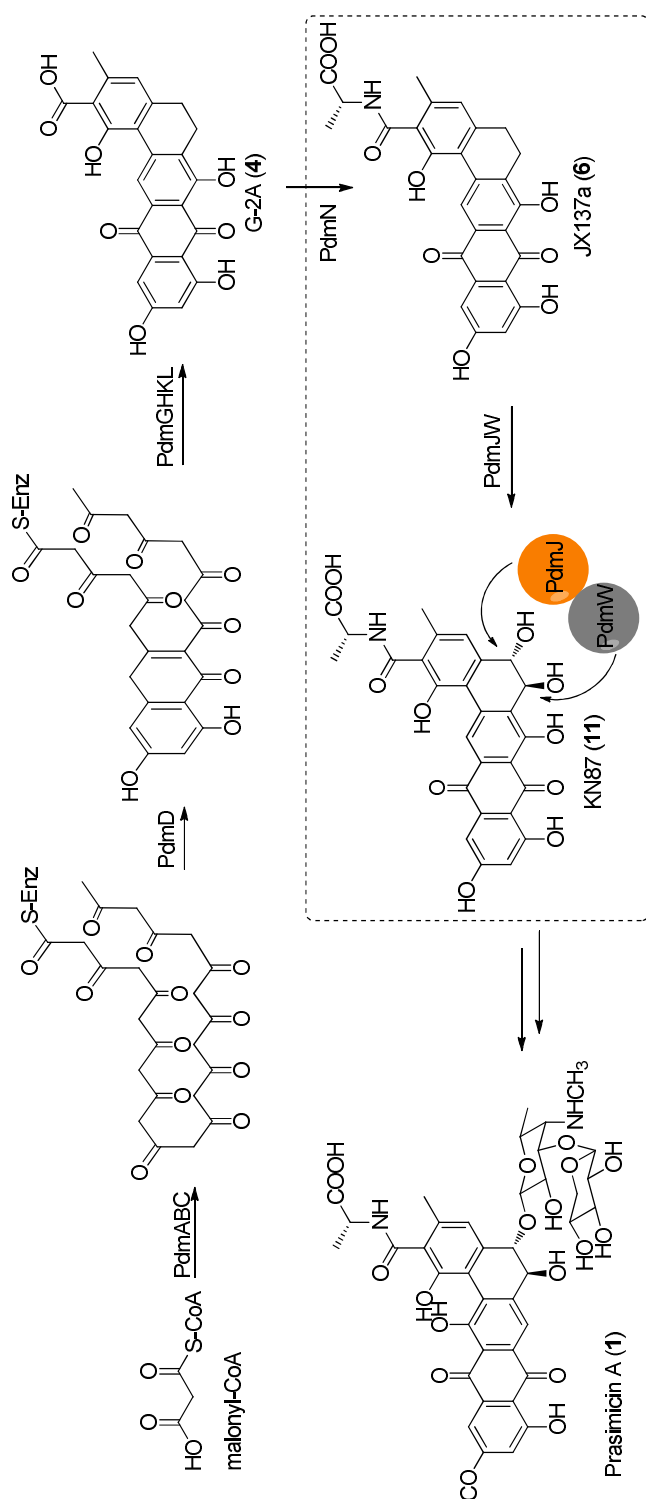


Figure 18. Biosynthetic pathway of 1. The steps studied in this work are boxed.

Compound **11** was identical to the PdmN-catalyzed biotransformation product of **9** in *S. coelicolor* CH999 (Figure 16, trace iv). Compound **11** was isolated from a scaled-up culture as an orange solid. Its molecular weight was found to be 535, according to the ion peaks, including  $[M-H]^-$  at  $m/z$  534.0 (Figure 36) and  $[M+H]^+$  at  $m/z$  536.1 in the ESI-MS spectra. It showed typical UV/Vis absorptions of pradimicins (Figure 37), thus suggesting that it is a pradimicin analogue. The  $^1H$  and  $^{13}C$  NMR (Figure 46 and Figure 47) spectra revealed that compared to **9**, **11** has extra proton and carbon signals including one methyl, one carboxyl, one methine and one NH group, which belongs to a D-alanine moiety introduced by PdmN. In the HMBC spectrum (Figure 49), the correlations of NH at  $\delta = 8.86$  (d,  $J=5.9$ , 1H) to C-16 at 167.5 and C-18 at 174.7, as well as H-19 at 1.39 ppm (d,  $J=7.2$ , 3H) to C-18 (Figure 14) confirmed that the amino acid was connected to C-16 to form an amide group (Figure 49). The  $^1H$ ,  $^1H$  ROESY correlations also supported the connection. Therefore, **11** was characterized as an alanylated analogue of **9**, which was named KN87 (Table 2) (Figure 46 to Figure 49). Compound **11** was found to be a major product in the culture of *S. coelicolor* CH999/pKN87 (Figure 17, trace v). It represents an important intermediate in pradimicin biosynthesis that can be further decorated by late tailoring enzymes, such as methyltransferases, oxygenases, and glycosyltransferases, to form the final structure of **1**.

Structural analysis of **10** and **11** confirmed that they were the products from the PdmN-catalyzed alanylation of **8** and **9**, respectively. It is apparent that all of the three studied tailoring enzymes (PdmJ, PdmW, and PdmN) can use **4** as a substrate. As shown in the HPLC traces of *S. coelicolor* CH999/pKN35 (Figure 13, trace i), *S. coelicolor* CH999/pKN92 (Figure 13, trace ii), and *S. coelicolor* CH999/pKN93 (Figure 17, trace ii),

PdmN can ligate the D-alanine moiety into **4** to form **6** efficiently, whereas PdmJ and PdmW are much less efficient in their corresponding hydroxylations. Different combinations of these tailoring enzymes generate various product profiles that provide important information about substrate specificity of these enzymes. Efficient production of **11** further confirmed that synergistic actions of biosynthetic enzymes are important for the assembly of **1**. Compounds **4–11** were tested for the antifungal activity against *Candida albicans* ATCC MYA-2876. However, none of these compounds showed inhibition of this fungus at the test concentrations (0.9–500 mg/mL). This is consistent with previous reports on the analogues of the aglycon pradimicinone, which further confirmed that the sugar moieties are essential for the biological activities of pradimicins.<sup>21, 22</sup>

### 3.5 Conclusions

Compound **1** is a promising lead compound for antiviral and antifungal therapeutics development. Combinatorial biosynthesis represents an effective approach to creating diverse analogues for drug development.<sup>23</sup> In this work, PdmW was characterized as the C-6 hydroxylase, and PdmN was found to accept different amino acid acceptor substrates. Moreover, this work demonstrated synergistic actions of two tailoring CYP enzymes in pradimicin biosynthesis (Figure 18). Together with our previous discovery on the collaboration of two cyclases and a monooxygenase in the early biosynthetic steps of **1**,<sup>17</sup> it can be concluded that the pradimicin biosynthetic enzymes have extensive synergistic actions and interactions during the biosynthetic process. Concerted actions of the PKS and three cyclases have also been reported in the resistomycin biosynthetic pathway to form the discoid ring structure.<sup>24</sup> Thus, we propose that synergistic actions of biosynthetic



enzymes are a common approach for type II polyketide biosynthetic pathways that consists of a series of discrete enzymes to achieve the accuracy and overall efficiency of the product assembly lines. Understanding of the functions of individual enzymes and their synergistic actions will facilitate rational and efficient combinatorial biosynthesis of new molecules for bioactivity screening.

### 3.6 Acknowledgements

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## CHAPTER 4

### THREE ENZYMES INVOLVED IN THE *N*-METHYLATION AND INCORPORATION OF THE PRADIMICIN SUGAR MOIETIES<sup>3</sup>

#### 4.1 Abstract

Pradimicins are antifungal and antiviral natural products from *A. hibisca* P157-2. The sugar moieties play a critical role in the biological activities of these compounds. There are two glycosyltransferase genes in the pradimicin biosynthetic gene cluster, *pdmS* and *pdmQ*, which are putatively responsible for the introduction of the sugar moieties during pradimicin biosynthesis. In this study, we disrupted these two genes using a double crossover approach. Disruption of *pdmS* led to the production of pradimicinone I, the aglycon of pradimicin A, which confirmed that PdmS is the *O*-glycosyltransferase responsible for the first glycosylation step and attaching the 4',6'-dideoxy-4'-amino-D-galactose or 4',6'-dideoxy-4'-methylamino-D-galactose moiety to the 5-OH. Disruption of *pdmQ* led to the synthesis of pradimicin B, revealing that this enzyme is the second glycosyltransferase that introduces the D-xylose moiety to the 3'-OH of the first sugar. Insertion of an integrative plasmid before *pdmO* might have interfered with the dedicated promoter, yielding a mutant that produces pradimicin C as the major metabolite, which suggested that PdmO is the enzyme that specifically methylates the 4'-NH<sub>2</sub> of the 4',6'-dideoxy-4'-amino-D-galactose moiety. Functional characterization of these sugar-

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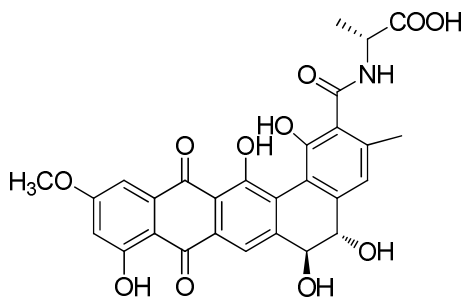
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decorating and -incorporating enzymes thus facilitates the understanding of the pradimicin biosynthetic pathway.<sup>1</sup>

## 4.2 Introduction

Pradimicins A–C (**1–3**) (Table 1) are aromatic polyketide natural products from the soil bacterium *A. hibisca* P157-2. Since their discovery in 1988, these molecules have been intensively studied. **1** is a promising lead compound due to its combined antifungal/antiviral properties. It was found to be active against a broad-spectrum of opportunistic and pathogenic fungi. This compound also interferes with the recognition of HIV-1 to its target cells. The mechanism of action of **1** emphasizes its lectin-like property in the presence of  $\text{Ca}^{2+}$  ions.<sup>2</sup> The moieties of **1** form a primary cavity with C-14 and C-15 from the benzo[ $\alpha$ ]naphthacenequinone and several hydroxyl groups of D-mannopyranoside.<sup>3</sup> Based on the intermolecular distance in the proposed model, it is believed that the free carboxyl group at C-18 of two molecules of **1** interacts with one  $\text{Ca}^{2+}$  ion.<sup>3</sup> Another study on the anticandidal mode of action was done with the semisynthetic pradimicin derivative, BMY-28864.<sup>4</sup> It was concluded that the sugar moieties of pradimicins, especially thomosamine or 4', 6'-dideoxy-4'-methylamino-D-galactose, were critical for sugar-recognition and involved in binding to the specific mannan. The pradimicin (*pdm*) biosynthetic gene cluster has previously been reported (NCBI accession number: EF151801.1). This reported gene cluster consists of 28 open reading frames.<sup>5</sup> Through combinatorial biosynthesis and heterologous expression, we have identified the enzymes that synthesize the pentangular intermediate G-2A, including PdmA, B, C, D, G, H, K, and L.<sup>6</sup> Two cytochrome P450 enzymes, PdmJ and PdmW, were functionally

characterized as the dedicated hydroxylases that specifically introduce a hydroxyl group to C-5 and C-6, respectively.<sup>4</sup> We have also identified PdmN as an amino acid ligase that introduces a D-alanine moiety at C-16. This enzyme was found to possess broad substrate and donor specificity.<sup>7, 8</sup> Synergistic actions of tailoring enzymes in pradimicin biosynthesis commonly exist to ensure an efficient assembly process. In this work, we show the functional characterization of three late tailoring enzymes, including PdmS, PdmQ and PdmO, using a homologous gene recombination approach. PdmS and PdmQ were characterized as the GTs that successively introduce the 4',6'-dideoxy-4'-methylamino-D-galactose/4',6'-dideoxy-4'-amino-D-galactose and D-xylose moieties to the structure, while PdmO is responsible for the *N*-methylation of 4',6'-dideoxy-4'-amino-D-galactose at 4'-NH<sub>2</sub>.



Pradimicinone I (**12**)

Figure 19. The structure of the pradimicin aglycon (**12**).

As in the pradimicin family, many other important antibiotics such as erythromycin naturally contain deoxysugar moieties attached to the aglycon. Because these sugar moieties often play a critical role in the bioactivities of these natural products, it is of great interest to identify the GTs responsible for transferring these deoxysugars to the aglycons.

The *pdm* biosynthetic gene cluster contains two putative GT genes, *pdmS* and *pdmQ*. Sequence analysis indicated that they belong to a family of GTs that catalyzes the final stage of the biosynthesis of glycosylated natural products such as vancomycin and chloroeremomycin.

### 4.3 Materials and Methods

#### 4.3.1 Primers and plasmid construction

To understand the role of PdmS in pradimicin biosynthesis, we designed two sets of primers to inactivate this gene using a double crossover approach. A 1482-bp left arm and a 1404-bp right arm were cloned from the genome of *A. hibisca* P157-2. These two fragments were ligated to the thermal sensitive plasmid pKC1139 between HindIII and XbaI as well as XbaI and EcoRI, respectively, to yield a disruption plasmid pKN82. The primers used and plasmids constructed in this work are shown in Table 9 and Table 11, respectively. Using a similar double crossover approach, we constructed a pKC1139-derived disruption plasmid pKN88 (Table 11) after the amplification of a 1224-bp left arm and a 1445-bp right arm.

To determine the function of PdmO, we constructed an integrative plasmid pKN99 (Table 11) in the pKC1139 vector and a 1.3-kb insert that contains the C-terminal portion of *pdmO* and N-terminal portion of *pdmS* (Figure 21). In addition, to confirm the double cross over and integration of the plasmid we constructed the primers-check specific for *pdmS* and *pdmQ*, primer 1 and 2 for *pdmO* (Table 9), and M13-47 and RV-M specific for the integrative vector pKC1139.

Table 9. Primers to clone the upstream and downstream region of each gene.

Primer	Sequence
<i>pdmS</i> -KO-left arm-HindIII-F	5'- <u>AAGCTT</u> CCCGTCTCGGAGGTCAGC-3'
<i>pdmS</i> -KO-left arm-XbaI-R	5'- <u>TCTAGAG</u> ACGTAGAGCCGTTCCAGG-3'
<i>pdmS</i> -KO-right arm-XbaI-F	5'- <u>TCTAGAT</u> CCTGCACGTCCAGACCG-3'
<i>pdmS</i> -KO-right arm-EcoRI-R	5'- <u>GAATT</u> CCCACCCAACCCCTAACCC-3'
<i>pdmS</i> -check-F	5'-ATGGCGAACATCCTGATCAG-3'
<i>pdmS</i> -check-R	5'-TCAGGCGATGACGGGACGGC-3'
<i>pdmQ</i> -KO-left arm-HindIII-F	5'- <u>AAGCTT</u> CAGTCAGCAAGCACAGCAGG-3'
<i>pdmQ</i> -KO-left arm-XbaI-R	5'- <u>TCTAGAC</u> GTGGGATTCGGCAGCAT-3'
<i>pdmQ</i> -KO-right arm-XbaI I-F	5'- <u>TCTAGAC</u> GCCACATCGTCTGCTGC-3'
<i>pdmQ</i> -KO-right arm-EcoRI-R	5'- <u>GAATT</u> CCGTCCACCGTCACCTCCC-3'
<i>pdmQ</i> -check-F	5'-ATGGCCGACATGCGGATCGT-3'
<i>pdmQ</i> -check-R	5'-CTAAACCCCAAAAGTGAACC-3'
<i>pdmO</i> -KO-HindIII-F	5'- <u>AAGCTT</u> CGTCCTGGAACGGCTCTA-3'
<i>pdmO</i> -KO-XbaI-R	5'- <u>TCTAGA</u> AAGGTTCGAAGGTGCACATGTC-3'
Primer 1	5'- CCGAGTACACCGACACGAT-3'
M13-47	5'-GAGCGGATAACAATTTACACAGG-3'
RV-M	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'
Primer 2	5'-CTTGTCGATGAACCGTTCCG-3'

#### 4.3.2 Intergeneric conjugation

*E. coli* ET12567 (pUZ8002) was transformed with pKN82, pKN88, and pKN99 separately as the conjugation donor. 100 mL of each culture of *E. coli* ET12567(pUZ8002)/pKN82, pKN88, and pKN99 were grown separately in LB broth to an OD<sub>600</sub> of 0.4-0.6 in the presence of 50 mg L<sup>-1</sup> apramycin, 50 mg L<sup>-1</sup> kanamycin, and 25 mg L<sup>-1</sup> chloramphenicol. The cells were then collected by centrifugation and washed twice with blank LB medium to remove the antibiotics, and then suspended in 0.1 mL of LB medium. *A. hibisca* spores were collected with 2-YT broth after growing for 15 days on YM medium at 30 °C. The *A. hibisca* spores were heat shocked at 50 °C for 10 min. Serial dilutions of the germinated spore suspension were made and 100 µL of each dilution was

mixed with an equal volume of *E. coli* ET12567 (pUZ8002)/ pKN82, pKN88, or pKN99 on MS plates (20 g L<sup>-1</sup> D-mannitol, 20 g L<sup>-1</sup> soya flower, 20 g L<sup>-1</sup> glucose, and 20 g L<sup>-1</sup> agar), which were grown for 12 h at 30 °C. After incubation, the plates were overlaid with 1 mL of sterile water containing 30 mg L<sup>-1</sup> nalidixic acid and 50 mg L<sup>-1</sup> apramycin, and incubated at 30 °C until the exconjugants appeared. Exconjugants were visible after 4–5 days and started producing spores in 8–10 days. Correct transformants of *A. hibisca*/pKN82, *A. hibisca*/pKN88, and *A. hibisca*/pKN99 were cultivated in YM broth (4 g L<sup>-1</sup> D-glucose, 4 g L<sup>-1</sup> yeast extract, and 10 g L<sup>-1</sup> malt extract) in the presence of 50 mg L<sup>-1</sup> apramycin at 28 °C in a rotary shaker at 28 °C, and then cultivated on ISP4 plates with 50 mg L<sup>-1</sup> apramycin at 37 °C for a period of 10 days to allow the plasmid to integrate into the genome.

Conjugants of *A. hibisca*/pKN82 and *A. hibisca*/pKN88 were picked to YM broth without any antibiotics and incubated for 10 days at 28 °C. The cultures were then spread onto ISP4 plates without antibiotics and grown for about 10 days at 28 °C<sup>9</sup>. The selection process for positive double crossover mutants was performed using a replica plate technique for colonies sensitive to 50 mg L<sup>-1</sup> apramycin. Positive colonies were cultivated in YM broth in a rotary shaker at 28 °C and were subjected to PCR screening using a set of *pdmS* and *pdmQ* specific primers (Table 9). The conjugants *A. hibisca*/pKN99 were selected on ISP4 medium with 50 µg mL<sup>-1</sup> apramycin at 37 °C. Integration of pKN99 into the genome of *A. hibisca* was confirmed by PCR analysis using genome- and vector-specific primers (Table 9).



#### 4.3.3 Large scale culture and purification

A seed culture of the mutant strain *A. hibisca*/pKN82 was cultivated in 50 mL of YM medium for 4 days at 28°C in a rotary shaker at 250 RPM. The seed culture was inoculated to 500 mL YM broth and cultivated at the same conditions for 10 days. The culture of the double crossover mutant of *A. hibisca*/pKN82 was centrifuged and the supernatant was subjected to Diaion HP-20 resin column chromatography the column was eluted with 0%, 25%, 50%, 75%, and 100% aqueous methanol (v/v) to yield five fractions. HPLC analysis revealed that the 75% fraction contained the target peak. This fraction was further separated by a reverse-phase Agilent 1200 HPLC (Agilent Zorbax SB-C18 column, 5 µm, 21.2 mm × 150 mm), eluted with 35% aqueous acetonitrile containing 0.1% formic acid, to yield 5.0 mg of **12**.

#### 4.3.4 LC-MS and NMR analysis

ESI-MS were acquired on an Agilent 6130 LC-MS HPLC (Agilent Zorbax SB-C18 column, 5 µm, 21.2 mm × 150 mm). 1D and 2D NMR spectra were recorded on a JEOL ECX-300 instrument.

### 4.4 **Results and Discussion**

#### 4.4.1 BLAST analysis

BLAST analysis revealed that PdmS (GenBank accession number ABK58688) is a putative GT that contains 437 aa. Two proteins with the highest homology include two proposed GTs from *Paenibacillus dendritiformis* (GenBank accession number WP\_006678995, 423 aa, 61% identity) and *Bacillus cereus* (GenBank accession number WP\_002061747, 416 aa, 59% identity). However, none of these are functionally

characterized. We next sought to identify the *O*-GT that transfers the D-xylose moiety. PdmQ (GenBank accession number ABK58687) is the second putative GT in the *pdm* gene cluster that contains 435 aa. BLAST analysis showed that it is homologous to many GTs that are proposed to catalyze the transfer of sugar moieties from activated donor molecules to specific aglycons. The closest homologs found in GenBank are two GTs from *Spirillospora albida* (GenBank accession number WP\_030163570, 414 aa, 61% identity) and *Thermomonospora curvata* (GenBank accession number WP\_012855060, 418 aa, 62% identity), respectively. Again, none of these homologs are identified.

The structure of **1** contains a 4',6'-dideoxy-4'-methylamino-D-galactose moiety, while that of **3** does not have the N-CH<sub>3</sub> group. A methyltransferase (MT) must be responsible for the methylation of this sugar moiety at the specific 4'-NH<sub>2</sub> group. There are three putative MTs in the *pdm* gene cluster, including *pdmF*, *pdmT* and *pdmO*. BLAST analysis suggested that PdmF and PdmT are two *O*-MTs, while PdmO is a putative *N*-MT. PdmO (GenBank accession number ABM21743.1) contains 238 aa. It is homologous to a number of class I MTs that are dependent of *S*-Adenosyl-L-methionine (SAM) such as the sugar *N,N*-dimethyltransferases DesVI from *Streptomyces venezuelae* (67% similarity and 53% identity)<sup>10</sup> and TylM1 from *Streptomyces fradiae* (66% similarity and 52% identity).<sup>11</sup> Both enzymes are involved in the biosynthesis of aminodeoxysugars. Thus, PdmO is a candidate enzyme that catalyzes the specific *N*-methylation of 4',6'-dideoxy-4'-amino-D-galactose.

Table 10.  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **12** (recorded in  $d_6$ -DMSO, 300 MHz).

No.	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1	-	150.9
2	-	127.1
3	-	137.4
4	7.04(s)	118.0
4a	-	140.9
5	4.22(bris)	71.5
6	4.22(bris)	72.4
6a	-	149.9
7	8.07(s)	115.6
7a	-	131.2
8	-	187.5
8a	-	110.1
9	-	164.8
10	6.87(bris)	106.9
11	-	166.0
12	7.21(bris)	107.6
12a	-	134.3
13	-	185.2
13a	-	115.4
14	-	156.8
14a	-	125.9
14b	-	113.7
15	2.30(s)	19.2
16	-	167.2
17	4.37(m)	47.7
18	-	174.2
19	1.30(d, J=6.9Hz)	16.9
11-OMe	3.89(s)	56.5
9-OH	12.8(s)	-
16-NH	8.50(d, J=6.5Hz)	-

#### 4.4.2 Plasmid construction

List of plasmids constructed in this study. The PCR amplification of the left and right arm for disruption were sequenced; before the construction of their respective integration plasmid.

Table 11. Plasmids designed to disrupt the genes *pdmS*, *pdmQ*, and *pdmO*.

Plasmid	Description	Reference
pKN64	Left arm for <i>pdmS</i> disruption in pJET1.2	This work
pKN63	Right arm for <i>pdmS</i> disruption in pJET1.2	This work
pKN73	Left arm for <i>pdmS</i> disruption in pKC1139	This work
pKN82	Both left and right arms for <i>pdmS</i> disruption in pKC1139	This work
pKN70	Left arm for <i>pdmQ</i> disruption in pJET1.2	This work
pKN71	Right arm for <i>pdmQ</i> disruption in pJET1.2	This work
pKN81	Left arm for <i>pdmQ</i> disruption in pKC1139	This work
pKN88	Both left and right arms for <i>pdmQ</i> disruption in pKC1139	This work
pKN68	A 1.3-kb fragment consisting of the C-terminal portion of <i>pdmS</i> and N-terminal portion of <i>pdmO</i> in pJET1.2	This work
pKN99	A 1.3-kb fragment consisting of the C-terminal portion of <i>pdmS</i> and N-terminal portion of <i>pdmO</i> in pKC1139	This work

#### 4.4.3 Confirmation of mutants by PCR and LC-MS analysis

*A. hibisca/pKN82*. As shown in Figure 22 (panel i), a 1.3-kb fragment of *pdmS* can be amplified from the genome of the wild type, while a disrupted gene *pdmS* with a length of 0.9 kb was cloned from the correct double crossover mutant. This confirmed that a 0.4-kb fragment of *pdmS* has been successfully deleted from the genome of *A. hibisca* P157-2. The  $\Delta$ PdmS mutant of *A. hibisca* P157-2 was then grown in YM medium for product analysis. The fermentation broth was centrifuged to separate the cells and supernatant, and the latter was injected into LC-MS for analysis. As shown in Figure 20, trace i, the wild type strain produces **1** as the major metabolite, with **2** and **3** as minor products depending on the culture time. In contrast, the  $\Delta$ PdmS mutant did not produce **1-3**. Instead, a new peak **12** (Table 2) was produced as a dominant product at 44 minutes (Figure 20, trace ii). The UV/Vis spectrum of **12** is similar to that of **1** (Figure 52), indicating that they have the same chromophore. Its molecular weight was found to be 549, according to the ion peaks

$[M-H]^-$  at  $m/z$  548 and  $[M+H]^+$  at  $m/z$  550. **12** in the ESI-MS spectra (Figure 53). This suggested that the strain *A. hibisca*/pKN82 has a  $\Delta$ PdmS and produced the pradimicin aglycon that has no sugar moieties.

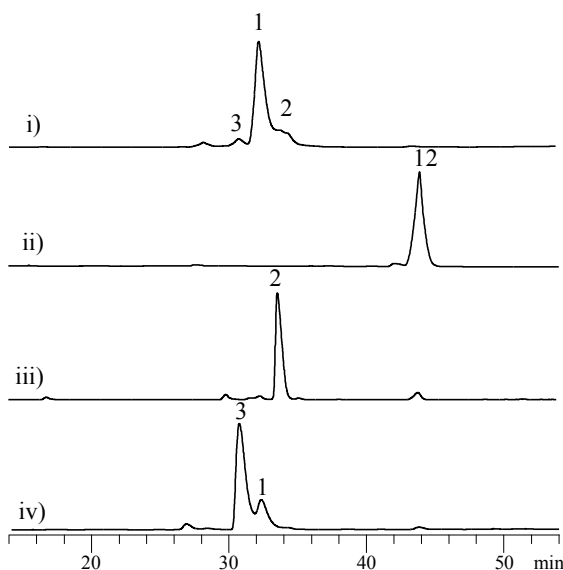


Figure 20. HPLC analysis of the products synthesized by the mutant strains. *A. hibisca*. (i), *A. hibisca*  $\Delta$ PdmS (ii), *A. hibisca*  $\Delta$ PdmQ (iii), and *A. hibisca*/pKN99 single crossover mutant (iv). Samples were eluted with 20–35% acetonitrile-water (containing 0.1% trifluoroacetic acid) over 40 minutes at 1 mL min<sup>-1</sup>.

Figure 22 (panel ii), the intact *pdmQ* gene is 1.3 kb, while its mutant *pdmQ* is 1.0 kb, confirming that a 0.3-kb fragment has been deleted from *pdmQ*. This mutant was grown in YM medium and the fermentation broth was analyzed by LC-MS. The double crossover mutant of *A. hibisca*/pKN88 did not produce **1** and **3**, but a dominant product with the same retention time as **2** (Figure 20, trace iii). Further analysis of the UV/Vis absorption spectra (Figure 52) and MS (Figure 53) confirmed that this product is indeed pradimicin B (**2**). Thus, disruption of PdmQ led to the production of a major product with only the 4',6'-

dideoxy-4'-methylamino-D-galactose moiety, which confirmed the function of this enzyme as the *O*-GT responsible for transferring the second sugar moiety, D-xylose. Compound **12** was observed as a minor product in this mutant, likely due to the incomplete glycosylation of this aglycon by PdmS.

The fermentation broth of *A. hibisca*/pKN99 in YM medium supplemented with 50 mg L<sup>-1</sup> apramycin was analyzed by LC-MS. As shown in Figure 20 (trace iv), this strain produced a major product **3** that is slightly polar than **1**. This compound has a UV/Vis spectrum (Figure 52) similar to that of **1**, while ESI-MS revealed that its molecular weight is 826 (Figure 53), 14 mass units smaller than **1**. It has the same retention time as that of pradimicin C (**3**). Thus, it can be concluded that *A. hibisca*/pKN99 produced **3** as the major product, with **1** as the minor product. Given the fact that **3** lacks the N-CH<sub>3</sub>, PdmO was deduced to be the dedicated *N*-MT in pradimicin biosynthesis that catalyzes the specific *N*-methylation of the 4',6'-dideoxy-4'-amino-D-galactose moiety. Integration of pKN99 into the genome of *A. hibisca* did not disrupt *pdmO* (Figure 21).

Consequently, the synthesis of **1** was still observed. However, insertion of a large fragment upstream of the intact *pdmO* may have influenced the function of the promoter that controls the expression of PdmO. This explains why **3** was generated as the major metabolite and is similar to the observations in our previous work on HerF in herboxidiene biosynthesis.<sup>12</sup> Also **12** was a minor metabolite in this mutant, which might be caused by the incomplete glycosylation of the aglycon.

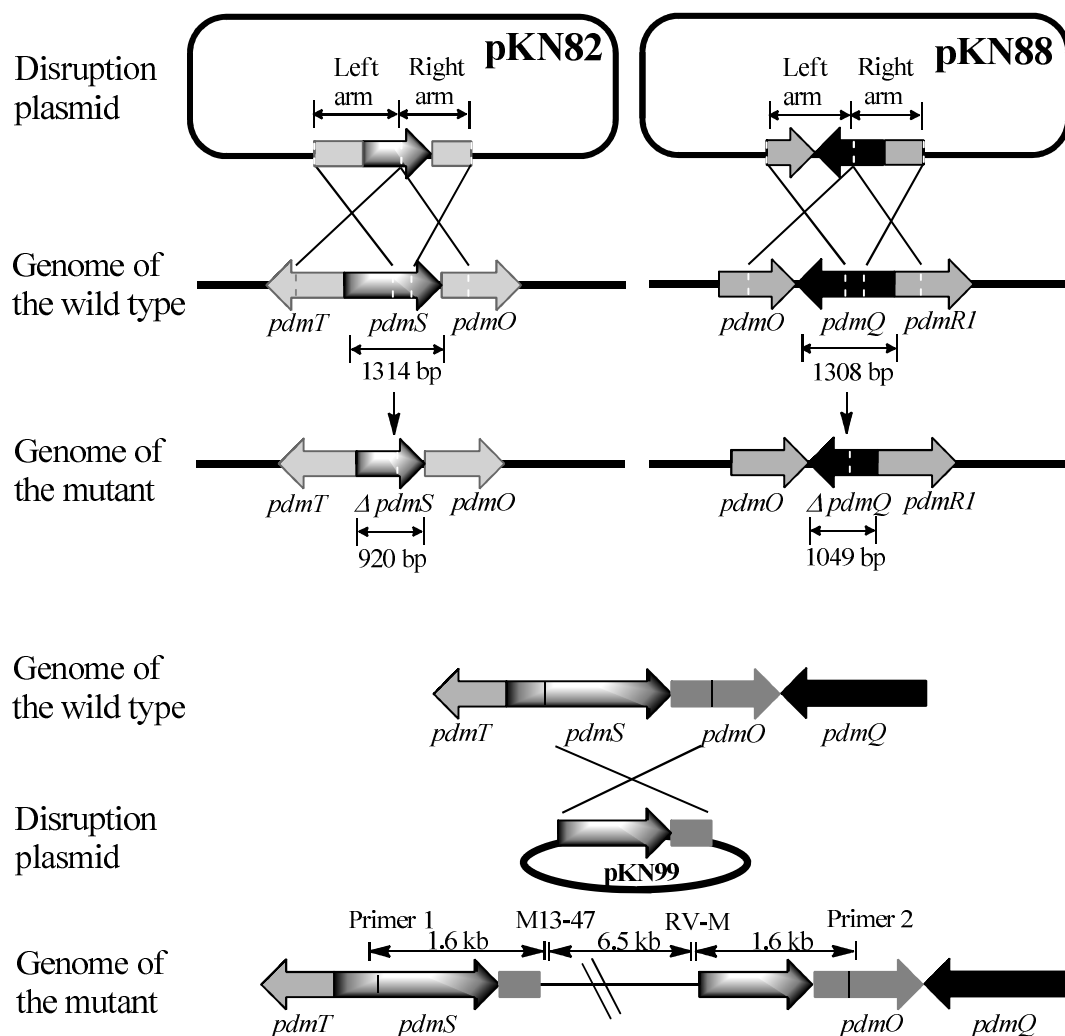


Figure 21. Disruption of *pdmS*, *pdmQ* and *pdmO* in *A. hibisca*. Double crossover and disruption gene strategies.

#### 4.4.4 Structure determination by NMR

The NMR spectra of **12** were recorded. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra indicated that there are only signals of the pradimicin aglycon in **12**, confirming that it is pradimicinone I. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR signals were assigned based on the 2D NMR spectra including HSQC, HMBC and ROESY, and are shown in Table 10. These data were identical with

those of reported for pradimicinone I <sup>13</sup> Production of **1–3** by *A. hibisca* revealed that pradimicins with one or two sugar moieties are naturally synthesized, suggesting that the two sugar moieties are successively introduced. Identification of the product of the  $\Delta$ PdmS mutant revealed the function of PdmS as the *O*-GT that is responsible for the introduction of the first sugar moiety, 4',6'-dideoxy-4'-amino-D-galactose or 4',6'-dideoxy-4'-methylamino-D-galactose.

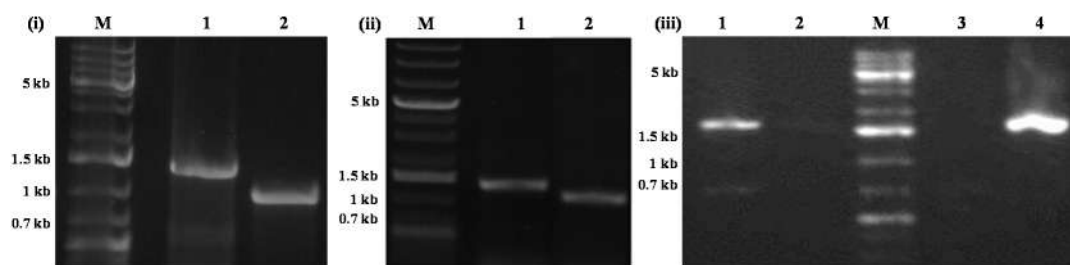


Figure 22. Verification of the correct mutants by PCR. (i) Amplification of pdmS from the wild type (1.3 kb) and mutant (0.9 kb). M: marker; 1: wild type; 2: *A. hibisca*/pKN82 double crossover mutant. (ii) Amplification of pdmQ from the wild type (1.2 kb) and mutant strain (1.0 kb). M: marker; 1: wild type; 2: *A. hibisca*/pKN88 double crossover mutant. (iii) Verification of the insertion of pKN99 into the genome of *A. hibisca*. M: marker; 1: primer 1/RV-M PCR product from the *A. hibisca*/pKN99 single crossover mutant; 2: primer 2/M13-47 PCR product from the *A. hibisca*/pKN99 single crossover mutant; 3: primer 1/primer 2 PCR product from the *A. hibisca*/pKN99 single crossover mutant; 4: primer 1/primer 2 PCR product from the wild type.

## 4.5 Conclusions

In summary, using a double crossover recombination approach, we disrupted two putative GT genes in the pradimicin biosynthetic gene cluster, leading to the production of pradimicin analogues with no or one sugar moiety. Based on the corresponding product of the mutants, PdmS and PdmQ were characterized as the GTs that are respectively responsible for the introduction of the first and second sugar moieties to the aglycon **12**



(Figure 23). By inserting a large gene fragment into the genome of *A. hibisca*, we also found that PdmO methylates the amino group of the 4',6'-dideoxy-4'-amino-D-galactose moiety in pradimicin biosynthesis (Figure 23). Unveiling the roles of these GTs and the *N*-MT further facilitates the understanding and rational engineering of the pradimicin biosynthetic pathway.

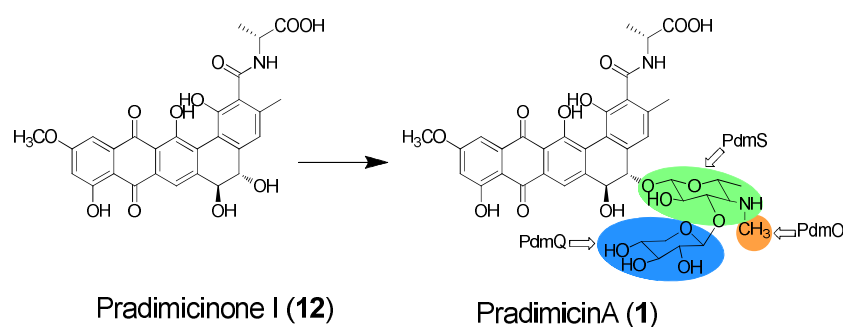


Figure 23. Visual conclusion of the last steps in the synthesis of **1**.

#### 4.6 Acknowledgements

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## CHAPTER 5

### TWO PUTATIVE *O*-METHYLTRANSFERASES: PdmT and PdmF

#### 5.1 Abstract

The sequence analysis of the gene cluster of pradimicin revealed two putative *O*-methyltransferase genes, *pdmT* and *pdmF*. However, the final structures of the members in the pradimicin family have only one-methoxy group. A mutant strain derived by double cross-over recombination knockout of *A. hibisca* was isolated for accumulating a new pradimicin analogue KN85 (**13**) and lacking detectable synthesis of pradimicin A-C. This mutant lacks the specific *O*-methyltransferase activity given by PdmT. The activity of PdmT was reconstituted *in vitro*, when the putative coding gene *pdmT* was expressed and purified from a heterologous *E. coli* system. Incubation of PdmT with SAM and **13** yielded 7-*O*-methyl-KN85 (**14**). Kinetic parameters of PdmT were obtained. These results established the function of PdmT as the 7-*O*-methyltransferase. Additional enzymes need to be discovered to understand how the methoxy group is removed to form the final structure of pradimicins A-C. Additionally, PdmF was revealed to be an *O*-methyltransferase that methylates the hydroxyl group at C-11 of pradimicins A-C. By using combinatorial biosynthesis, a series of genes was co-expressed in a heterologous host. The function of PdmF was confirmed by LC-MS analysis of the products of the engineered strains as well as from the *in vitro* reaction of PdmF with KN87 (**11**), which generated a new methylated metabolite (**17**).

## 5.2 Introduction

### 5.2.1 Methyltransferases in natural product biosynthesis

PKSs and fatty acid synthases use condensation of simple carboxylic acid monomers common to synthesize their metabolic products.<sup>1</sup> Besides the essential catalytic enzymes, several auxiliary functional (tailoring) enzymes add a variety of distinct chemical groups to the backbone residues of the secondary metabolites. This post-PKS modification results in increased structural diversity of small molecules, which radically affects the molecules' biological activity, solubility, and binding properties. The tailoring enzymes vary in accordance with each antibiotic gene cluster; they include MTs, GTs, cytochromes P450, CYP, oxidases, amino acid ligases, or catalysts for other reactions.

MTs are a large group of enzymes that methylate diverse substrates, including signaling and host defense compounds, pigments, prosthetic groups, cofactors, cell membrane, cell wall components, and xenobiotics.<sup>2</sup> They can be divided into several subclasses based on their structural characteristics. Class I is the most common type MT, characterized by a Rossmann fold for binding the co-substrate SAM.<sup>3</sup> Class II MTs contain a SET domain,<sup>3, 4</sup> and class III MTs are associated with the cell membrane.<sup>5</sup> All natural product-MT (NPMT) structures elucidated belong either to class I<sup>3</sup> or class III.<sup>4</sup> MTs can also be grouped into different types according to different substrates in methyl transfer reactions. These types include protein MTs, DNA MTs or macromolecular MTs, MTs (NPMT), and non-SAM dependent MTs. More often, MTs are categorized based on the electron-rich methyl-accepting atom, including O (54% of EC subclass),<sup>6, 7, 8</sup> N (23%),<sup>8, 9</sup> C (18%),<sup>6</sup> S (3%),<sup>2, 6, 10, 11</sup> or halide (2%),<sup>10, 12, 13</sup> and some NPMTs methylate more than one type of acceptor atom.<sup>14</sup> NPMTs can methylate on the polyketide aglycon or on the

sugars components, the sugar can be methylated either before or after transferring to the aglycon.

An example of a post-PKS sugar modification is the reaction catalyzed by the *N*-MT PdmO, in which the thomosamine (4'-amino-4',6'-dideoxy-D-galactose) is modified to finally synthesize 4',6'-dideoxy-4'-methylamino-D-galactose in pradimicin A and B.<sup>15</sup> Another example occurs during oleandomycin production, when OleY MT is involved in the biosynthesis of one of its sugars, L-oleandrose. L-olivose is first transferred to the oleandolide aglycone, which is then converted into L-oleandrose by the OleY MT.<sup>39</sup> The biosynthesis of the anthracycline antitumor daunomycin has two MTs involved in its synthesis; *dauC* catalyzes the SAM-dependent methylation of aklanonic acid to aklanonic acid methyl ester. While *dauK* coded for carminomycin 4-*O*-methyltransferase, which catalyzes the transfer of a methyl group from SAM to the 4-*O*-position of carminomycin to form daunomycin.<sup>16</sup> Also, the synthesis of tetracenomycin C requires three SAM-dependent methylations by TcmN, TcmO, and TcmP. TcmN has a second function: it uses its N-terminal portion in the cyclization of a polyketide, while the C-terminal portion contains the methyltransferase activity.<sup>17</sup> TcmP lacks the SAM binding motif and its amino acid sequence is different from other SAM-dependent enzymes.<sup>18</sup> The *temP* gene contains codons infrequently used in *Streptomyces spp.*, the codons TTA and TTT,<sup>19</sup> positioned in the C-terminal part of the ORF, are not present in any of the other ORFs of the entire TCM C cluster.<sup>20</sup> In the biosynthesis of herboxidine, HerF is the methyl transferase in the last step of the pathway.<sup>21</sup>

SAM is the standard methyl donor for MTs; however, other methyl donors are seen in nature. The overall mechanism to transfer a methyl group is a S<sub>N</sub>2-like nucleophilic

attack where the methionine sulfur assists as the nucleophile that carries the methyl group to the substrate (Nu:). SAM is converted to *S*-adenosyl-L-homocysteine (SAH) through this reaction. The cleavage of the SAM-methyl bond and the release of the substrate-methyl bond (Nu-CH<sub>3</sub>) happen nearly simultaneously (Figure 24).

NPMTs are attractive targets for protein engineering due to their variety of substrates and the broad substrate selectivity that some NPMTs have. However, more studies on the regeneration of SAM in a metabolic engineering context are required in order to engineer NPMTs.<sup>22, 23, 24</sup> Regiospecific methylation is challenging, it often requires elaborated steps for the addition of protecting groups and does not always produce high yields.

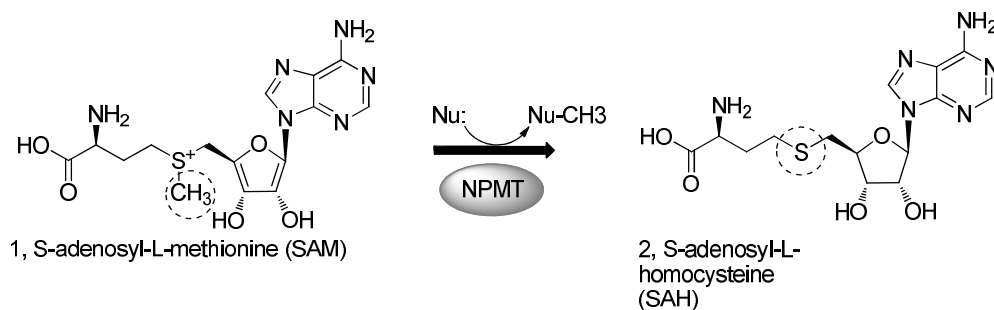


Figure 24. Reaction mode of SAM-dependant NPMTs. NPMTs use SAM as a methyl donor (dashed) for transfer to a methyl acceptor (Nu:). The methylated product forms (Nu-CH<sub>3</sub>) and *S*-adenosyl-L-homocysteine (SAH) is produced and it is a potent inhibitor of SAM-dependent MTs.

The NPMTs in type II polyketide biosynthetic pathways vary in the number of amino acid residues, usually ranging from 200 to 500, corresponding to the molecular weight of 25-55 kDa; however, for modular PKS, methyltransferase (MT) domains have been described<sup>25</sup> for PKS type I and the hybrid NRPS (non-ribosomal peptide synthetase)/type I PKS.<sup>26</sup> Almost all NPMTs currently isolated share  $\alpha$ -helices and  $\beta$ -

strands alternating along their polypeptide chain. Homologous sequence studies of SAM-dependent NPMTs have found conserved motifs among these proteins.<sup>27, 28, 29, 30</sup> The regions associated with SAM binding are related to motifs I – VI.<sup>27, 31, 32</sup>

Sequence analysis of the two MTs studied in this chapter has been conducted by alignment with MTs from the biosynthetic pathways of other angucyclic antibiotics such as rubromycin, griseorhodin, and fredericamycin. PdmT (340 aa) was proposed to be a MT based on the amino acid sequence homology to TcmO (339 aa) from *S. glaucescens* and GrhL (343 aa) from *Streptomyces sp.* JP95. The putative function of PdmF (342 aa) was likewise proposed due to its homology to TcmN (494 aa) from *S. glaucescens* and FdmN (353 aa) from *S. griseus*.<sup>33</sup> This study established the functional characterization of PdmT and PdmF in the pradimicin biosynthetic pathway.

### 5.3 Materials and Methods

#### 5.3.1 General

ESI-MS spectra were acquired on an Agilent 6130 LC-MS HPLC (Agilent Zorbax SB-C18 column, 5  $\mu$ m, 4.6  $\times$  250 mm). 1D and 2D NMR spectra were recorded on a JEOL instrument (300 MHz). *E. coli* XL1-Blue (Agilent) was used as a host for general cloning and *E. coli* ET12567(pUZ8002) was used as a donor strain for intergeneric conjugation *E. coli*/*Actinomadura*.<sup>34</sup> *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3)-RIL were used as the protein expression hosts. All strains of *E. coli* were grown in LB medium at 250 rpm and 37°C in the presence of appropriate antibiotics (50  $\mu$ g mL<sup>-1</sup>): ampicillin, kanamycin, or/and chloramphenicol. *A. hibisca* P157-2 strain was obtained from the American Type Culture Collection (ATCC 53557) and was grown in YM broth or YM agar.

The commercial vectors pJET1.2 (Fermentas) and pCR-Blunt (Life technologies) were used as the cloning vectors. The temperature sensitive *E. coli-Streptomyces* shuttle vector (Apr<sup>r</sup>) used was pKC1139.<sup>35</sup> The vector pET28a(+) was used for protein expression under the T7 promoter. Isolation of plasmid DNA from *E. coli* was performed with a GeneJET™ Plasmid Miniprep Kit (Fermentas) by following the manufacturer's instructions. Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs.

Table 12. Designed PCR primers for knockout and protein expression.

Primer	Sequence
<i>pdmT</i> - left arm-HindIII-F	5'- <u>AAGCTT</u> CCCCCG ACACC CTCAA CAT -3'
<i>pdmT</i> - left arm-XbaI-R	5'- <u>TCTAGA</u> CGGCG ACTTCT TCAAC GAC -3'
<i>pdmT</i> -right arm-XbaI-F	5'- <u>TCTAGA</u> CGGTG TAGGT GGGCT TGG -3'
<i>pdmT</i> -right arm-EcoRI-R	5'- <u>GAATTC</u> GGTCT GGACG TGCAG GAAC -3'
<i>pdmT</i> -NdeI-F	5'-AAC <u>ATATG</u> ACGGT GACCG GCACA CC-3'
<i>pdmT</i> - EcoRI -R	5'-AAGA <u>ATTC</u> TCAGC GCTTG CGCGC GATG-3'
<i>pdmF</i> - left arm-HindIII-F	5'- <u>AAGCTT</u> GCGCA TGTTT CTCGA CTACG -3'
<i>pdmF</i> - left arm-XbaI-R	5'- <u>TCTAGA</u> GTCGC GGTGG CTGTC GTC -3'
<i>pdmF</i> -right arm-XbaI-F	5'- <u>TCTAGA</u> CTACC TGCTG AAAGC GATCC -3'
<i>pdmF</i> -right arm- EcoRI -R	5'- <u>GAATTC</u> CCGAC CGAGT ACCAG ACCC -3'
<i>pdmF</i> -NdeI-F	5'- AAC <u>ATATG</u> ATGACC GAACC GGAAG GAC -3'
<i>pdmF</i> -EcoRI-R	5'- <u>GAATTC</u> TCAGA TGGGG CGGCA CTCCA -3'

### 5.3.2 Primers and cloning

To investigate the function of the putative *O*-methyltransferases, PdmT and PdmF, an approach to disrupt the genes by double-crossover homologous recombination was engineered. The primers were designed to clone the upstream and downstream part of each gene to knockout. Each pair of homologous fragment would then be ligated into the



HindIII/EcoRI sites of the thermo sensitive vector pKC1139 to constitute the disruption plasmids. Moreover, specific primers for cloning the gene fragments were designed and are shown in Table 12.

### 5.3.3 Intergeneric conjugation approach

The procedure to disrupt the genes *pdmT* and *pdmF* in *A. hibisca* through double crossover recombination<sup>34</sup> was mentioned in detail in our previous publication.<sup>15</sup>

### 5.3.4 Combinatorial biosynthesis approach for *pdmF*

The plasmids in Table 13 were used for the co-expression of five combinatorial cassettes of genes previously constructed<sup>36, 37</sup> in the expression vector pRM5 (*tsr*). The gene *pdmF* was site-specifically integrated into the genome of *S. coelicolor* using the integrative vector pSET152 (Apr<sup>r</sup>) (pJX156). Each pair of plasmids was introduced into the protoplast<sup>34</sup> of *S. coelicolor* through PEG-mediated transformation<sup>34</sup>. After the regeneration of the protoplasts, the correct apramycin and thiostrepton resistant colonies were selected and picked to grow on R5 agar supplemented with 50 mg L<sup>-1</sup> apramycin and 50 mg L<sup>-1</sup> thiostrepton for a period of 12-15 days at 28°C. Each co-transformed strain was separately extracted with ethyl acetate:methanol:acetic acid (89:10:1) and dried in vacuo. The extracts were resuspended in 100% DMSO and analyzed by LC-MS (Agilent Zorbax SB-C18 column, 5 µm, 4.6 × 250 mm eluted with a linear gradient method (10 to 90% acetonitrile in water containing 0.1% trifluoroacetic acid over 25 min).

### 5.3.5 Protein expression and purification

*Methyltransferase PdmT*. For the expression of PdmT the corresponding gene was cloned from the genome of *A. hibisca*, using the pair of primers (*pdmF*-NdeI-F and *pdmF*-EcoRI-R) in Table 12 and ligated into the cloning vector pJET 1.2. After confirmation of

the correct amplification by DNA sequencing the gene *pdmT* was excised and ligated into the NdeI/EcoRI restriction sites of the expression vector pET28a(+) to yield the plasmid pKN122. The expression of pKN122 was performed by *E. coli* BL21 (DE3) and cultured on LB agar containing 50  $\mu\text{g mL}^{-1}$  kanamycin at 37 °C for 16 hours. 500  $\mu\text{L}$  of an overnight culture of BL21 (DE3)/pKN122 in LB broth was inoculated into 500 mL of LB supplemented with 50  $\mu\text{g mL}^{-1}$  kanamycin and cultivated at 37 °C in a rotary shaker at 250 RPM. When the OD<sub>600</sub> reached 0.4 - 0.6 (in about 4 hours) the culture was induced with 0.1 mM IPTG and expressed at 18 °C for 16 hours. The N-His<sub>6</sub>-tagged PdmT was purified by Ni-NTA chromatography and fractions collected were analyzed by SDS-PAGE. Protein quantification was determined with the Coomassie (Bradford) Protein Assay Kit from Thermo Scientific.

*Methyltransferase PdmF.* The gene *pdmF* was amplified from the genomic DNA of *A. hibisca* using the primers in Table 12 and cloned into the integrative pSET152 vector to yield the plasmid pJX156. DNA sequencing of the PCR product was performed to confirm correct amplification of the target gene. The gene *pdmF* was excised from pJX156 and ligated into the NdeI/EcoRI restriction sites of the vector pET28a(+) to yield the plasmid pKN1.

pKN1 was chemically transformed into the expression system *E. coli* BL21 (DE3)-RIL and cultured on LB agar containing 50  $\mu\text{g mL}^{-1}$  kanamycin and 25  $\mu\text{g mL}^{-1}$  chloramphenicol at 37 °C for 16 hours. 500  $\mu\text{L}$  of an overnight culture of BL21 (DE3)-RIL/pKN1 in LB broth was inoculated into 50 mL of LB supplemented with 50  $\mu\text{g mL}^{-1}$  kanamycin and 25  $\mu\text{g mL}^{-1}$  chloramphenicol and cultivated at 37 °C in a rotary shaker at 250 RPM. When the OD<sub>600</sub> reached 0.4 - 0.6 the culture was induced with 0.2 mM IPTG

and expressed at 28 °C for 16 hours. The *N*-His<sub>6</sub>-tagged PdmF was purified by Ni-NTA chromatography and fractions collected were analyzed by SDS-PAGE. Protein quantification was determined with the Coomassie (Bradford) Protein Assay Kit from Thermo Scientific.

Table 13. Plasmids used for combinatorial biosynthesis approach for PdmF.

Plasmid pairs	Genes	Products
pJX120 <sup>37</sup> + pJX156	<i>pdmABCDGHKL + F</i>	4
pKN93 <sup>36</sup> + pJX156	<i>pdmABCDGHKLN + F</i>	4, 6
pKN35 <sup>36</sup> + pJX156	<i>pdmABCDGHKLJ + F</i>	4, 5, 15
pKN92 <sup>36</sup> + pJX156	<i>pdmABCDGHKLW + F</i>	4, 8, 16
pKN87 <sup>36</sup> + pJX156	<i>pdmABCDGHKLJNW + F</i>	4, 6, 9, 11, 17

### 5.3.6 Kinetics

*In vitro reactions of PdmT.* The enzyme PdmT was reacted with KN85 (**13**) (Table 2), the accumulated intermediate in the  $\Delta$ *pdmT* mutant strain *A. hibisca*/pKN85. A standard *in vitro* reaction contained 75 mM Tris-HCl buffer (pH 7.5), 2 mM SAM, 0.05 mM of substrate (**13**), and 10.4  $\mu$ M PdmT in a total volume of 100  $\mu$ L. The reactions were quenched by heating inactivation of the enzyme at 90°C for 5 min. To obtain the kinetic parameters, different concentrations of the substrates (ranging from 0.025 to 0.8 mM) were used. After centrifugation (15,000 rpm for 10 min) of the quenched reactions the supernatants were analyzed by LC-MS (Agilent Zorbax SB-C18 column, 5  $\mu$ m, 4.6  $\times$  250 mm) eluted with a linear gradient method (30 to 40% acetonitrile in water containing 0.1% trifluoroacetic acid over 40 min).

*In vitro reaction of PdmF.* The enzyme PdmF was reacted with KN87 (**11**). Reactions contained 100 mM Tris-HCl buffer (pH 7.5), 1.22 mM SAM, 3  $\mu$ M PdmF, and

0.05 mM substrate **11**. Reactions were started by the addition of PdmF at 30 °C. After 2 h, the 100  $\mu$ L reaction mixtures were stopped by adding 50  $\mu$ L of methanol, which were then vortexed and centrifuged at 15,000 rpm for 10 min. The control was performed with boiled PdmF. The supernatants were analyzed by LC-MS (Agilent Zorbax SB-C18 column, 5  $\mu$ m, 4.6  $\times$  250 mm) eluted with a linear gradient method (10 to 60% acetonitrile in water containing 0.1% trifluoroacetic acid over 25 min).

### 5.3.7 Compound purification and structure determination by NMR

*Compound KN85 (13)*. The culture seed of *A. hibisca*/pKN85 was grown in 50 mL of YM broth, in a 250 RPM rotary shaker at 28 °C for 4 days. 10 mL of the culture seed of *A. hibisca*/pKN85 was inoculated to 500 mL of YM broth and cultivated for 15 days at 28 °C in a rotary shaker at 250 RPM. The *A. hibisca*/pKN85 culture was centrifuged at 4,000 RPM for 8 minutes to separate the supernatant from the cell debris. The supernatant was then loaded onto an adsorbent Diaion HP-20 resin in an open column. The loaded column was washed with 4 volumes of deionized water, to remove the broth media and free molecules, and eluted with 25%, 50%, 75%, and 100% aqueous methanol (v/v) to yield five fractions. Each fraction was analyzed by HPLC. The fraction that contained the purest **13** was dried in vacuo and dissolved in [*d*<sub>6</sub>] DMSO for further <sup>1</sup>H NMR analysis in a JEOL ECX-300 instrument.

*Compound 7-O-methyl-KN85 (14)*. The compound **14** (Table 2) was directly purified by HPLC (Agilent Zorbax SB-C18 column, 5  $\mu$ m, 4.6  $\times$  250 mm) from the *in vitro* reactions of PdmT/KN85 with constant method (30% acetonitrile in water containing 0.1% trifluoroacetic acid). **14** was dried in vacuo and dissolved in DMSO-*d*<sub>6</sub> for further <sup>1</sup>H NMR analysis on a JEOL ECX-300 instrument.

## 5.4 Results and Discussions

To confirm the function of PdmT and PdmF, the upstream and downstream regions of *pdmT* and *pdmF* were cloned with the right and left arm primers engineered in Table 12. Each PCR product was ligated into the cloning vector pJET 1.2 for further DNA sequencing to confirm correct amplification (Table 14). Each sequenced result was then aligned with its homologous sequence using the DNAssist Software Version 2.0. After confirmation of their correct amplification, the left and right arms were excised and subsequently ligated into pKC1139 between the HindIII/XbaI and XbaI/EcoRI sites, respectively. The partial and final disruption plasmids are listed in Table 14. The genes *pdmT* and *pdmF* were ligated into the expression vector pET28a(+) to yield the final plasmids pKN122 and pKN1, respectively (Table 14).

Table 14. Plasmids for double cross over recombination and protein expression.

Plasmid	Arms/Genes	Vector	Reference
pKN61	Upstream of <i>pdmT</i>	pJET1.2	This work
pKN62	Downstream of <i>pdmT</i>	pJET1.2	This work
pKN76	Upstream of <i>pdmT</i>	pKC1139	This work
pKN85	Upstream & downstream of <i>pdmT</i>	pKC1139	This work
pKN117	Mutant <i>pdmT</i>	pJET1.2	This work
pKN118	<i>pdmT</i>	pJET1.2	This work
pKN122	<i>pdmT</i>	pET28a(+)	This work
pKN65	Upstream of <i>pdmF</i>	pJET1.2	This work
pKN66	Downstream of <i>pdmF</i>	pJET1.2	This work
pKN77	Upstream of <i>pdmF</i>	pKC1139	This work
pKN80	Upstream & downstream of <i>pdmF</i>	pKC1139	This work
pJX156	<i>pmdF</i>	pSET152	This work
pKN1	<i>pdmF</i>	pET28a(+)	This work

The plasmids pKN85 and pKN80 were separately introduced into wild type *A. hibisca* by intergeneric conjugation through *E. coli* ET12567.<sup>34</sup> The generated apramycin-

resistant strains *A. hibisca*/pKN85 and *A. hibisca*/pKN80 appeared after 4 days of culture on MS agar at 28°C. For each strain, after plasmid-integration into the genomic DNA at 37 °C and double crossover recombination at 28 °C, the correct mutant phenotype was apramycin sensitive. The mutant strain *A. hibisca*/pKN85 was successfully obtained after several LC/MS analysis of the extract of potential mutant cultures. The mutant *A. hibisca*/pKN85 did not produce **1** but a less polar metabolite with maximum UV/Vis absorptions spectrum (240, 290, and 510 nm) (Figure 26 B, trace ii) that are different from those of **1** (230, 300, 460 nm) (Figure 26 B, trace i). This new metabolite, KN85 (**13**) (Figure 26 A, trace ii), has a molecular weight of 565, 275 amu smaller than **1**. The <sup>1</sup>H NMR spectra of **13** was recorded. It was determined that **13** has the same structure as the previously reported pradimicinone II (PMN II)<sup>38</sup> (Figure 25 A) (Table 2). The proton signals were assigned based on the 1D and 2D NMR spectra and are shown in Figure 25 A (Table 15).

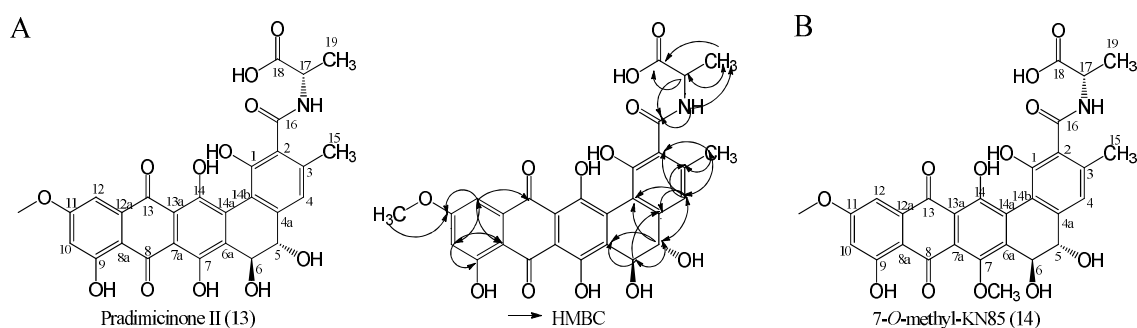


Figure 25. Structure determination of compounds **13** and **14** by NMR. (A) The HMBC and ROESY correlation of **13**. (B) Structure of **14**.

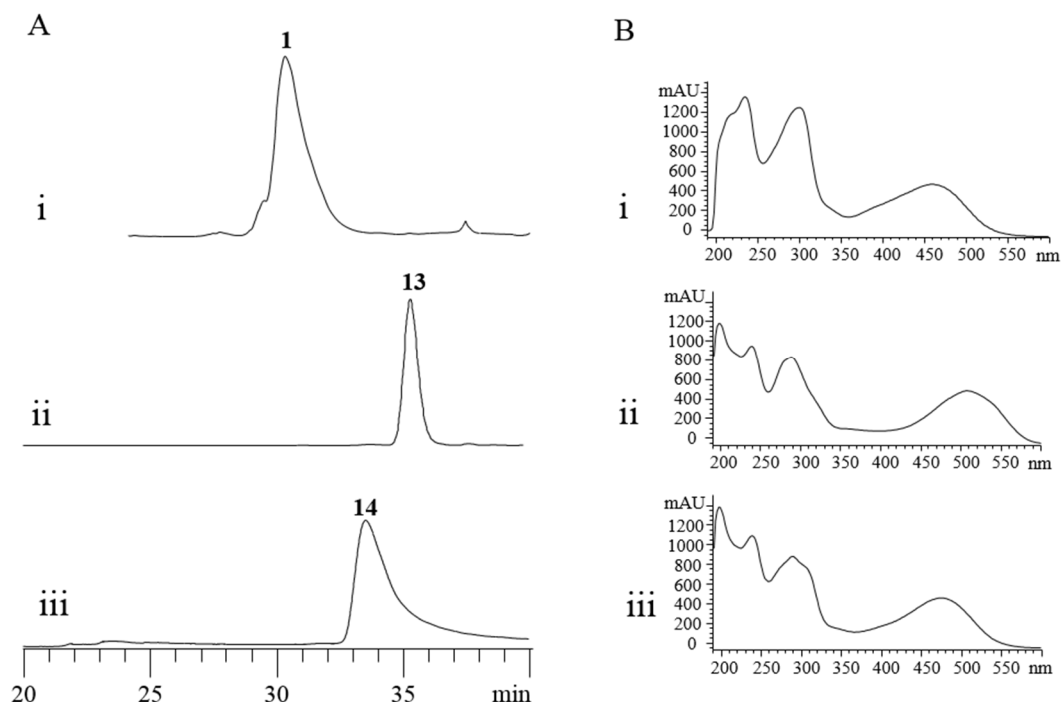


Figure 26. LC/MS chromatograms and absorbance of compounds **1**, **13**, and **14**. (Ai) Wild type *A. hibisca* culture presenting the production of **1** and (Bi) its characteristic UV/Vis spectrum (230, 300, 460 nm). (Aii) Mutant type *A. hibisca*/pKN85 showing its major product **13** and its (Bii) unique UV/Vis spectrum (240, 290, and 510 nm). (Aiii) HPLC profile for the *in vitro* conversion of **13** to **14** by PdmT, (Biii) typical UV/Vis spectrum of **14** (240, 290, and 475 nm).

Identification of the product of the  $\Delta pdmT$  mutant indicated the function of PdmT as the *O*-MT that is responsible for the methylation of the hydroxyl group at C-7. To confirm the deletion of the gene *pdmT*, the genotype of the mutant *A. hibisca*/pKN85 was analysed by PCR of the gene *pdmT* and compared to the amplification of the same gene from the wild type *A. hibisca* using the primers *pdmT*-NdeI-F/*pdmT*-EcoRI-R (Table 12). The PCR product obtained from the mutant strain,  $\Delta pdmT$ , was 386-bp shorter (Figure 27 B, line 1) than *pdmT* amplified from the wild type strain (1017-bp) (Figure 27 B, line 2). The amplification of  $\Delta pdmT$  was ligated into the pJET 1.2 vector to yield the plasmid

pKN117. Sequencing of the plasmid and its alignment with the *pdmT* sequence from NCBI (Accession number EF151801.1) confirmed that *A. hibisca*/pKN85 lacked a portion of the gene *pdmT*.

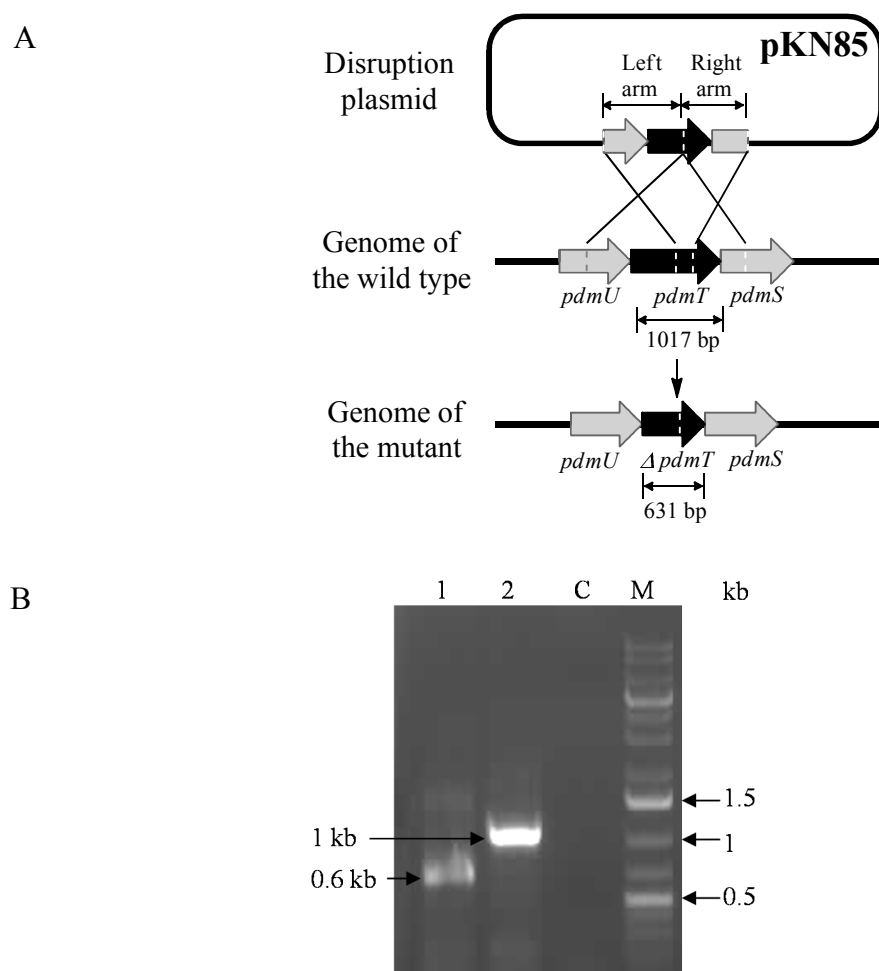


Figure 27. Construction and verification of the  $\Delta$ *pdmT* mutant strain. (A) Design of the plasmid for double crossover knockout of *pdmT*. (B) Line 1: Amplification of the gene *pdmT* from the mutant strain *A. hibisca*/KN85 (631 bp); line 2: Amplification of the gene *pdmT* from *A. hibisca* (1017 bp); C: Negative control; M: marker.

To further understand the function of PdmT, the gene *pdmT* was expressed (37.1 KDa) in the soluble form with the induction of 0.1 mM IPTG at 16 °C in a rotary shaker at 250 RPM. The *N*-His<sub>6</sub>-tagged protein was purified by Ni-NTA chromatography at 4 °C



with a yield of 9.46 mg/L (Figure 28). To investigate its function, PdmT was reacted with **13** at 30 °C. As shown in Figure 26 A, trace iii, the LC-MS results showed that all the substrate was converted to a more polar compound that has a different UV/Vis absorption spectrum (240, 290, 475 nm) and a molecular weight of 14 amu larger than **13** (Figure 26A, ii). These results strongly suggested the methylation of **13**, while the position of methylation was unknown. The product, **14**, was purified and subjected to  $^1\text{H}$  NMR for the determination of its structure (Table 15) (Figure 50) (Figure 51). Unexpectedly, we uncovered that the hydroxyl group at C-7 was methylated by PdmT (Figure 25 B). **13** can be efficiently converted to **14** by PdmT through *O*-methylation, confirming that this enzyme is a dedicated *O*-methyltransferase (Figure 29). We also obtained the kinetic parameters of PdmT,  $k_{cat}$ : 27.7 min $^{-1}$ ,  $K_M$ : 6.8 mM,  $k_{cat}/K_M$ : 4.1 min $^{-1}$ mM $^{-1}$ .

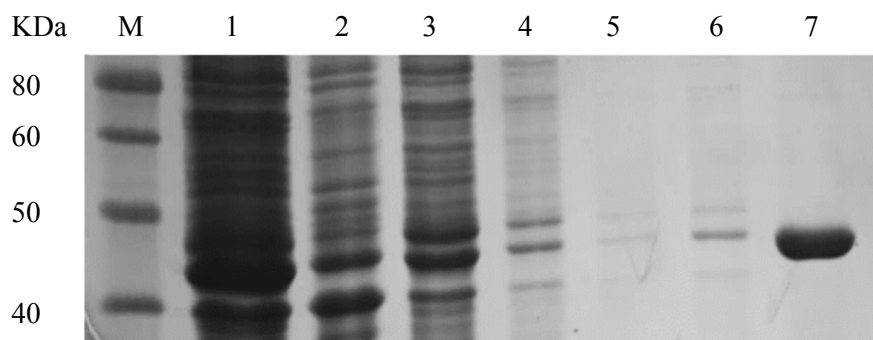


Figure 28. SDS-PAGE analysis of the expression and purification of PdmT. M: protein ladder, 1: soluble fraction, 2: insoluble fraction, 3: percolation, 4: elution with buffer A, 5: elution with buffer A and 10 mM imidazole 6: elution with buffer A and 25 mM imidazole, 7: Elution with 250 mM imidazole.

Thus, PdmT has been characterized as an *O*-methyltransferase involved in the biosynthesis of pradimicins. But because the final structures of pradimicins do not have

this methoxy group at C-7, we propose that **14** is an intermediate for further demethoxylation by other enzymes found in the gene cluster of pradimicin.

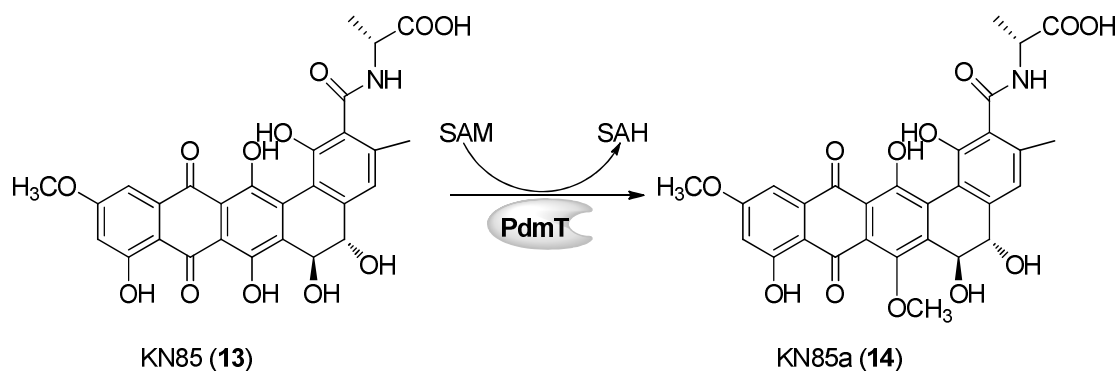


Figure 29. Proposed reaction catalyzed by PdmT.

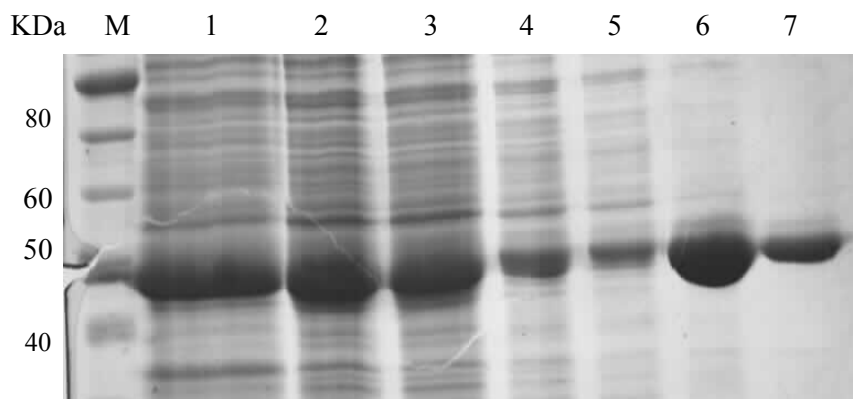


Figure 30. SDS-PAGE analysis of the expression and purification of PdmF. M: protein ladder, 1: soluble fraction, 2: insoluble fraction, 3: percolation, 4: elution with buffer A, 5: elution with buffer A containing 10 mM imidazole 6: elution with buffer A containing 25 mM imidazole, 7: elution with buffer A containing 250 mM imidazole.

The mutant strain *A. hisisca*/pKN80 underwent the same steps as the mutant strain *A. hisisca*/pKN85. However, we failed to obtain a  $\Delta pdmF$  mutant strain. We then took an alternative approach to study the function of PdmF. By using a combinatorial biosynthesis approach, the gene *pdmF* was co-expressed with five different cassettes of genes that

synthesize the backbone structure of pradimicins (Table 13). The products were analyzed by LC/MS. The strains that produced any pradimicin intermediates without a hydroxyl group at C-5 or C-6 (**4** and **6**) did not yield any 11-*O*-methylated products.

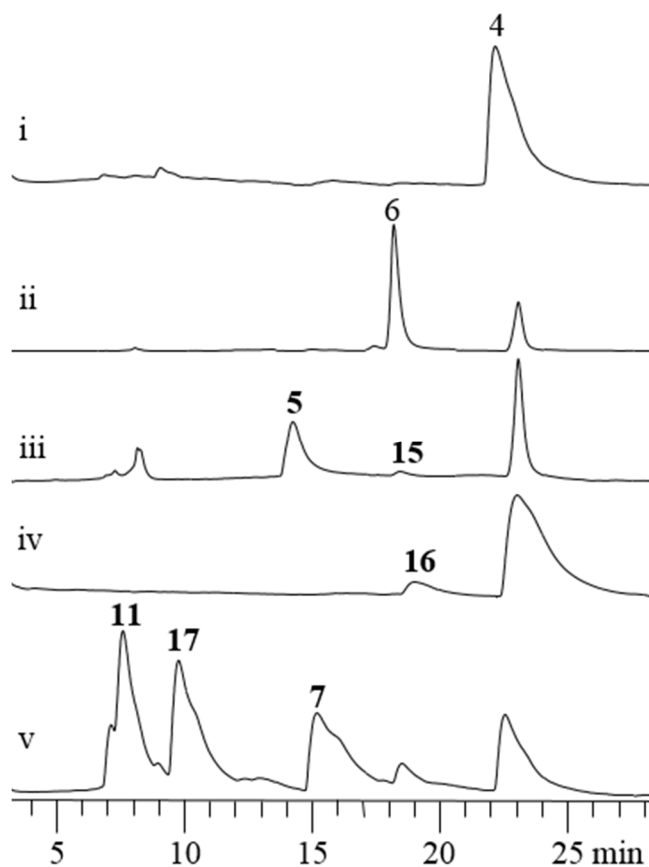


Figure 31. LC/MS analysis of new pradimicin analogues obtained by combinatorial biosynthesis. (i) Extract of strain *S. coelicolor*/pJX120 + pJX156. (ii) Extract of strain *S. coelicolor*/pKN93 + pJX156. (iii) Extract of strain *S. coelicolor*/pKN35 + pJX156. (iv) Extract of strain *S. coelicolor*/pKN92 + pJX156. (v) Extract of strain *S. coelicolor*/pKN87 + pJX156.

Table 15. The NMR data of KN85 (**13**) and 7-*O*-methyl-KN85 (**14**) (recorded in DMSO-*d*<sub>6</sub>, 300 MHz).

Position	$\delta_{\text{H}}$ KN85	$\delta_{\text{C}}$ KN85	$\delta_{\text{H}}$ 7- <i>O</i> -methyl-KN85
1	-	154.3	-
2	-	128.0	-
3	-	137.3	-
3-CH <sub>3</sub>	-	-	2.26 (3H, s)
4	6.73 (s)	123.2	6.46 (1H, s)
4a	-	139.9	-
5	4.46 (1H, brs)	71.2	4.32 (1H, brs)
6	5.06 (1H, brs)	62.9	4.94 (1H, brs)
6a	-	136.9	-
7	-	155.2	-
7-OH	13.03 (s)	-	-
7-OCH <sub>3</sub>	-	-	3.78 (3H, s)
7a	-	113.2	-
8	-	187.8	-
8a	-	110.2	-
9	-	164.1	-
9	12.34 (s)	-	-
10	6.73 (1H, brs)	106.1	6.68 (1H, brs)
11	-	166.0	-
11-OCH <sub>3</sub>	3.91 (3H, s)	56.3	3.87 (3H, s)
12	7.27 (1H, brs)	106.8	7.06 (1H, brs)
12a	-	136.6	-
13	-	183.5	-
13a	-	111.2	-
14	-	153.9	-
14a	-	136.7	-
14b	-	116.5	-
15	2.30 (3H, s)	18.9	-
16	-	167.5	-
16-NH	8.49 (1H, d, J=6.2)	-	8.79 (1H, brs)
17	4.34-4.42 (1H, m)	47.8	4.35 (1H, m)
17-CH <sub>3</sub>	-	-	1.21 (3H, brs)
18	-	174.2	-
19	1.35(3H, d, J=7.2)	17.1	-

The expression of PdmF (37.01 KDa) in the soluble form was achieved with the induction of 0.2 mM IPTG at 25 °C in a rotary shaker at 250 RPM. The *N*-His<sub>6</sub> tagged protein was purified by Ni-NTA chromatography at 4 °C with a yield of 26.39 mg L<sup>-1</sup> (Figure 30). To investigate the function of this putative methyltransferase, PdmF was reacted with **11** at 30 °C. As shown in Figure 32, trace i, the LCMS results showed that about 40% of the substrate **11** (MW 535) was converted to a less polar compound **17** (MW 549) (Figure 33C). Compound **17** has a similar UV/Vis absorption spectrum to **11** and 14 amu larger than **11**. The methylation product **17** strongly suggested that PdmF is an *O*-methyltransferase, also this result matches the result found in the combinatorial biosynthesis experiments (Figure 31, trace vi) where **11** is modified by PdmF *in vivo* to a compound with the same molecular weight (549), same UV/Vis absorption and same retention time as **17** (Figure 33).

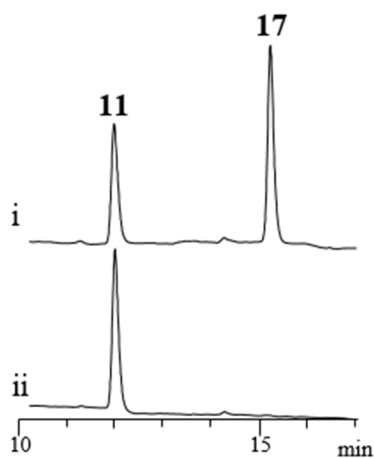


Figure 32. LC/MS analysis of PdmF *in vitro* reaction. (i) Methylation of **11** into **17**. (ii) Standard of **11**. HPLC conditions: 5 to 90% acetonitrile in water containing 0.1% trifluoroacetic acid over 30 min (Agilent Eclipse Plus-C18 column, 5  $\mu$ m, 4.6  $\times$  250 mm).

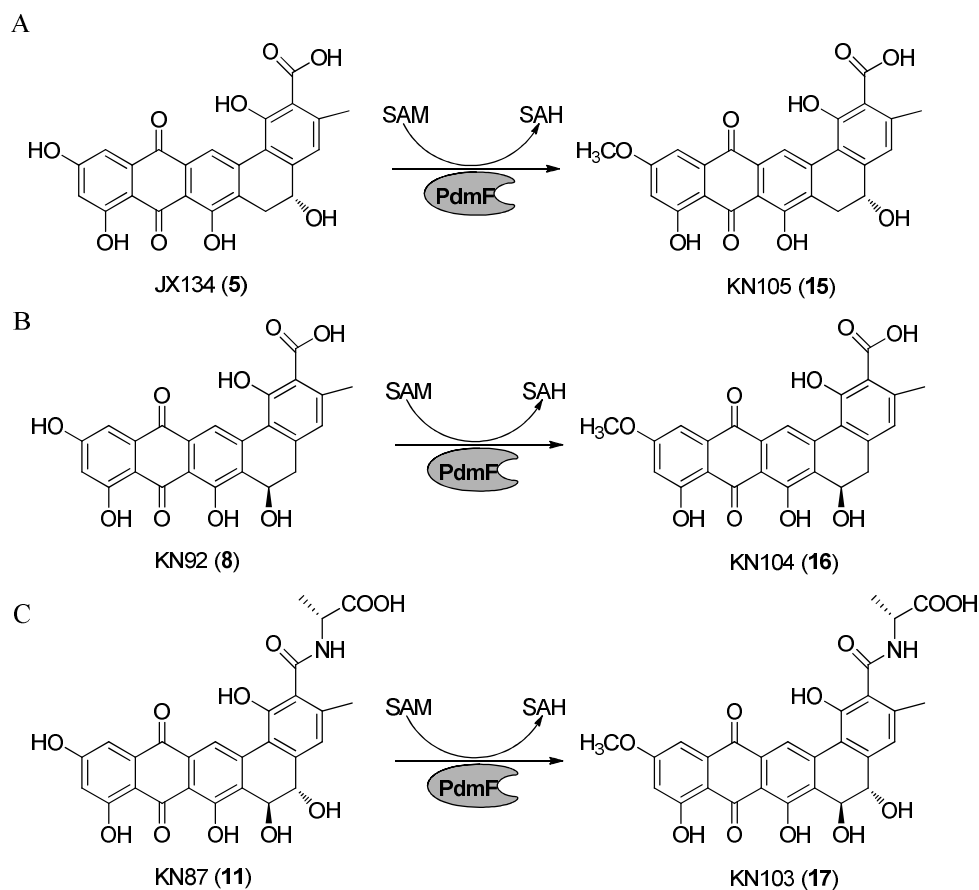


Figure 33. Proposed methyl transfer reactions from SAM by PdmF.

## 5.5 Conclusions

Our results revealed that PdmT is an *O*-methyltransferase that uses SAM as a methyl donor. The  $\Delta pdmT$  strain produced the metabolite **13**, which in turn was used as substrate for *in vitro* reactions with PdmT. As expected, PdmT methylated **13** at C-7 to form **14**. On the other hand, PdmF was revealed to be the enzyme that reacted with the hydroxyl group at C-11 to form the methoxy group found in the final structures of pradimicins.

We failed to obtain the  $\Delta pdmF$  strain; however, we tested the substrate specificity by co-expressing *pdmF* with different sets of pradimicin biosynthetic genes in *S. coelicolor*. Our results showed that new methylated metabolites were synthesized when PdmF was expressed in the system and only the intermediates that were mono- or dihydroxylated at position C-5 and/or C-6 accepted the methyl group. Additionally, the result from the *in vitro* reaction of PdmF and the intermediate **11** yielded a methylated compound, **17**, with the same characteristics as the molecule found in our first approach when **11** and PdmF were synthesized *in vivo*. Although the structure of **17** has not been determined by NMR spectroscopy, we are certain that PdmF is responsible for the methylation at C-11 in the synthesis of pradimicins. These results allowed us to characterize PdmT and PdmF as SAM-dependent *O*-methyltransferases.

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## CHAPTER 6

### CONCLUSIONS AND RECOMMENDED FUTURE RESEACH

#### 6.1 Conclusions

To summarize our findings, the reconstruction of the late steps of the pradimicin biosynthetic pathway was possible through the interaction of genetic concepts and engineering strategies. Eight enzymes that modify the core structure of pradimicins have been studied in this work, including cytochrome P450 hydroxylases (PdmJ and PdmW), amino acid ligase (PdmN), *O*- and *N*-methyltransferases (PdmF, PdmT, and PdmO), and *O*-glycosyltransferases (PdmS and PdmQ). Our findings functionally characterized and described the interactions of the mentioned enzymes, biosynthesized pradimicin precursors, while explaining the biosynthetic pathway of the antifungal and antiviral pradimicins.

The enzymes PdmJ and PdmW were characterized as cytochrome P450 hydroxylases that catalyze the incorporation of two hydroxyl groups at C-5 and C-6, respectively. Our *in vitro* studies revealed that PdmJ hydroxylates C-5 in **4** to form **5**. The PdmJ enzyme has narrow substrate specificity and substrate inhibition was detected for the first time in type II polyketide pathways, as well as competitive inhibition by **6**. The *in vivo* studies were performed in *E. coli* BL21(DE3) that expressed both PdmJ and GDH. The bioconversion of **4** to **5** was achieved in a higher yield by feeding substrate **4** to the induced fermentation broth of the *E. coli* strain. Moreover, this reaction was done in 2 days, rather than 8 days that is required for *S. coelicolor* CH999/pJX134 to produce **5**. The bioconversion approach by *E. coli* BL21(DE3)/pKN29 strain can be used to obtain **5** as a starting molecule for further modifications to generate novel pradimicin analogues. Additionally, PdmW was confirmed to be the cytochrome P450 hydroxylase responsible

for the hydroxyl group at C-6 of **4**. It was demonstrated that the co-expression of PdmJ and PdmW significantly improved the hydroxylation steps. Thus, synergistic actions of the two tailoring cytochrome P450 enzymes; as well as interactions with PdmN in pradimicin biosynthesis were established. We believe that synergistic actions of biosynthetic enzymes are common in type II polyketide biosynthetic pathways. PdmN is an amino acid ligase that accepts a variety of substrates including **4**, **8**, and **9** and ligates a D-alanine moiety to the C-16 carboxyl group. In addition, the enzymes PdmS and PdmQ were characterized as the *O*-glycosyltransferases responsible for the introduction of the first and second sugar moieties to the aglycon of pradimicin (**12**). Besides, we confirmed that PdmO is the *N*-methyltransferase that adds a methyl group to the amino group of the 4',6'-dideoxy-4'-amino-D-galactose moiety in pradimicins. PdmT and PdmF were characterized as *O*-methyltransferase enzymes that use SAM as a methyl donor. *In vitro* studies revealed that PdmT is responsible for the methylation of the hydroxyl group at C-7, which needs further enzymatic removal to form the aglycon of pradimicins. Additionally, PdmF was found to be the enzyme that reacted with the hydroxyl group at C-11 to form the methoxy group in the final structures of pradimicins.

All our results together provide useful information for understanding and engineering the late steps in the biosynthetic pathway of pradimicins. New pradimicin analogues may be further generated for better drug candidates by rationally manipulating this pathway.

## 6.2 Recommended Future Research

Future studies need to focus on the removal of the methoxy group at C-7. Because demethoxylation reactions are not common in natural product biosynthetic pathways, it

will be valuable to discover the enzyme that demethoxylates C-7. Since collaboration and synergy of enzymes have been observed in the pathway of pradimicins, it will not be a surprise that this demethylation is performed by two or more enzymes working together. There are a few remaining putative genes in the gene cluster that have not been studied yet, which may be further investigated for the enzymes involved in the C-7 demethoxylation.

Additionally, by analyzing the mechanism of action of pradimicins and the pool of pradimicin precursors generated in this study, we believe that **12** and **13** might be good candidates for further modifications of their structure due to the presence of D-alanine, the hydroxyl group at C-14, the estimated production yield, and the easy extraction and purification. The fact that these two precursors lack the sugar moiety, directs glycosylation to C-5 or C-6. Since PdmS and PdmQ are classified now as *O*-GTs, structural analysis of these enzymes might help in the modification of their binding site to make them accept other sugar molecules and thus get modified pradimicins. Likewise, structural analysis for the other enzymes characterized in this studied may reveal important data about the substrate binding and catalytic sites, especially the enzymes with broad substrate specificity.

From a different perspective, these molecules are endowed with pigmentation. It is possible that they could be modified to work as natural dyes, similar to  $\lambda$ -actinorhodin, a natural dye from another soil bacterium, *Streptomyces coelicolor*.

## **APPENDICES**

## **APPENDIX A**

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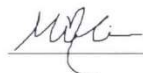
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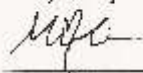
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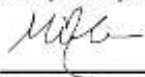
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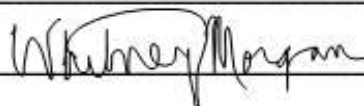
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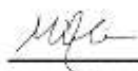
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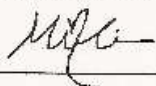
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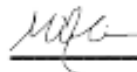
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## APPENDIX B.

Data of compound **10** (KN102):  $^1\text{H}$ -NMR data ( $\text{DMSO-}d_6$ ,  $\delta$  in ppm, J in Hz): 9.02(1H, s, H-14), 7.12(1H, s, H-12), 6.61(1H, s, H-4), 6.45(1H, s, H-10), 5.22(1H, brs, H-6), 4.20(1H, s, H-17), 2.93-3.02 (1H, m, H-5a), 2.72-2.80(1H, m, H-5b), 2.23(3H, s, H-15), 1.27(3H, s, H-19); ESI-MS:  $m/z$  518.2 $[M-H]^-$ .

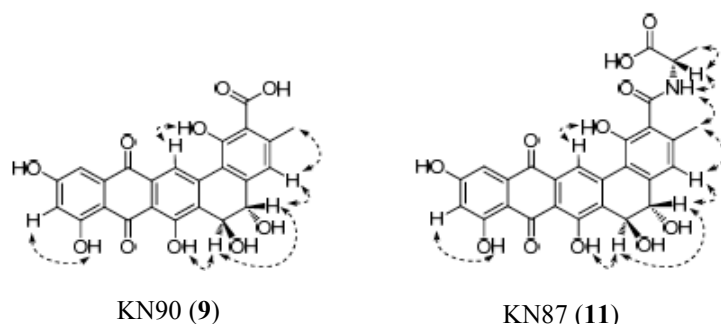


Figure 34. Key  $^1\text{H}$ - $^1\text{H}$  ROESY correlations for **9** and **11**.

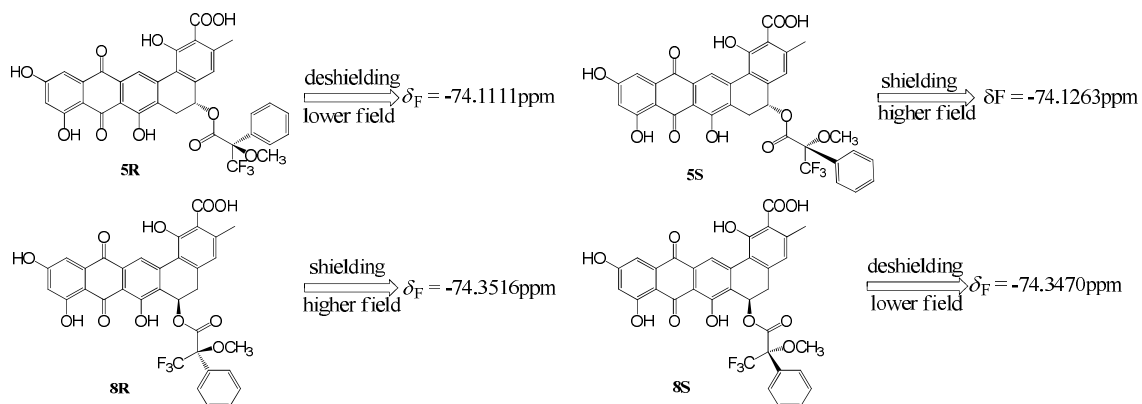


Figure 35. Determination of the absolute configurations of **5** and **8**.  $^{19}\text{F}$  NMR of Mosher's method.  $\delta_F$  (ppm) values in pyridine- $d_5$  are shown.

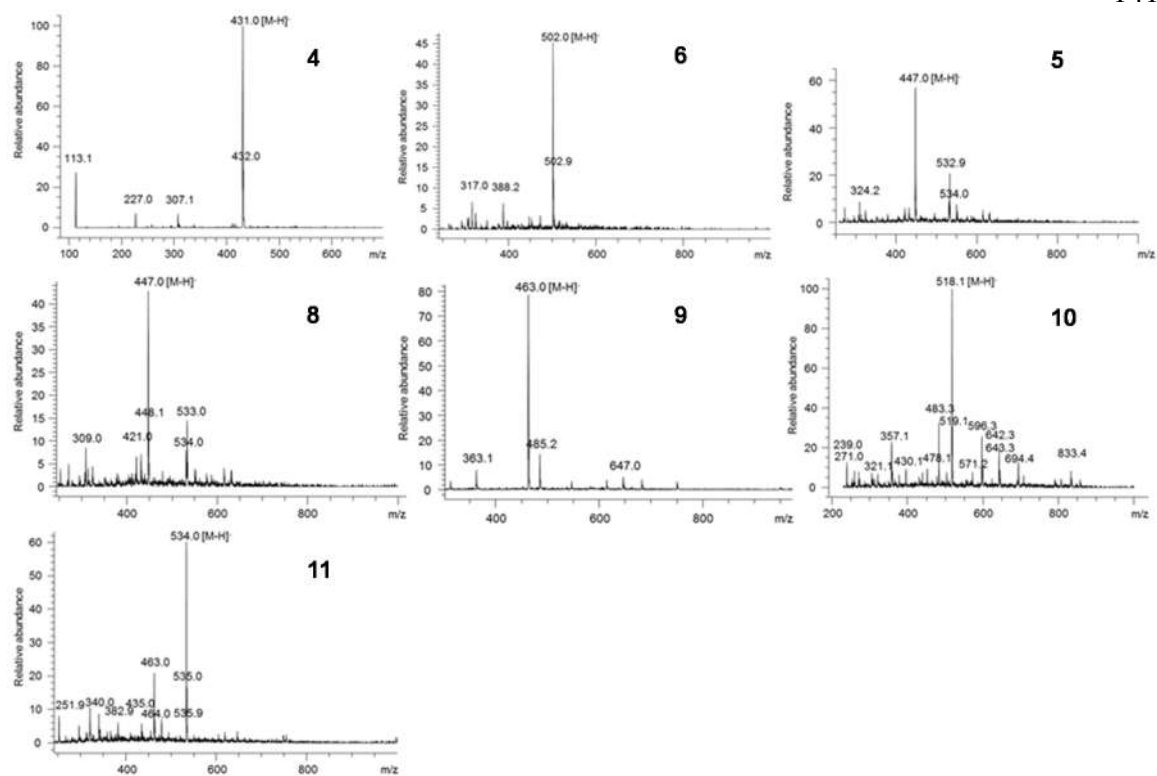


Figure 36. ESI-MS (-) spectrum of compounds 4-11.

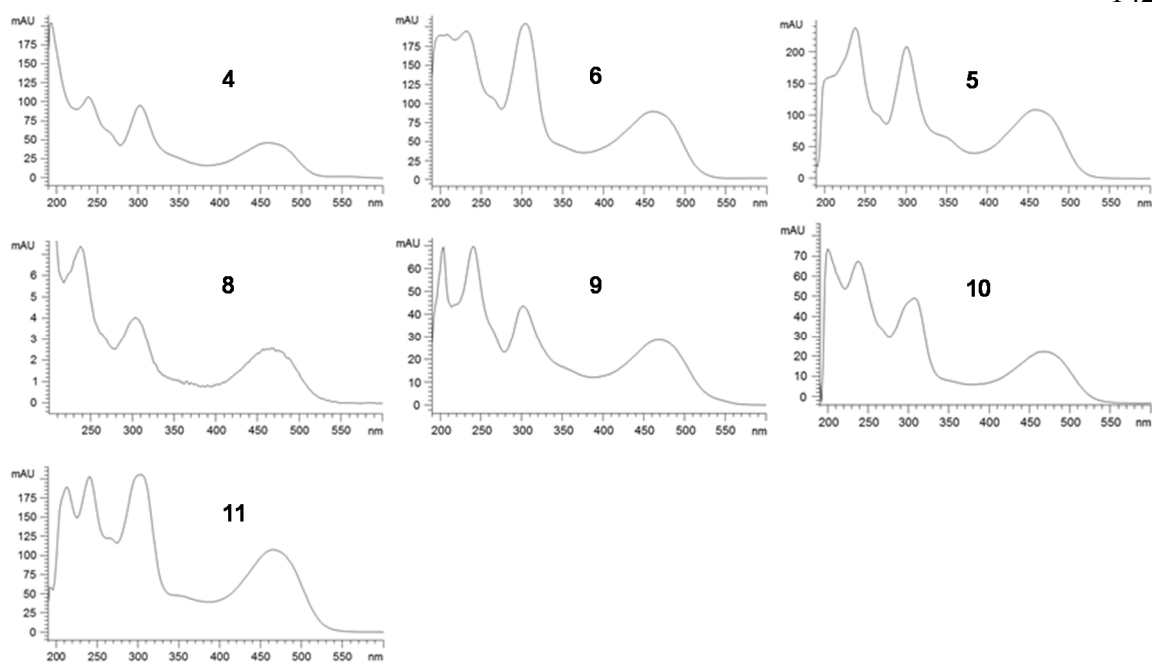
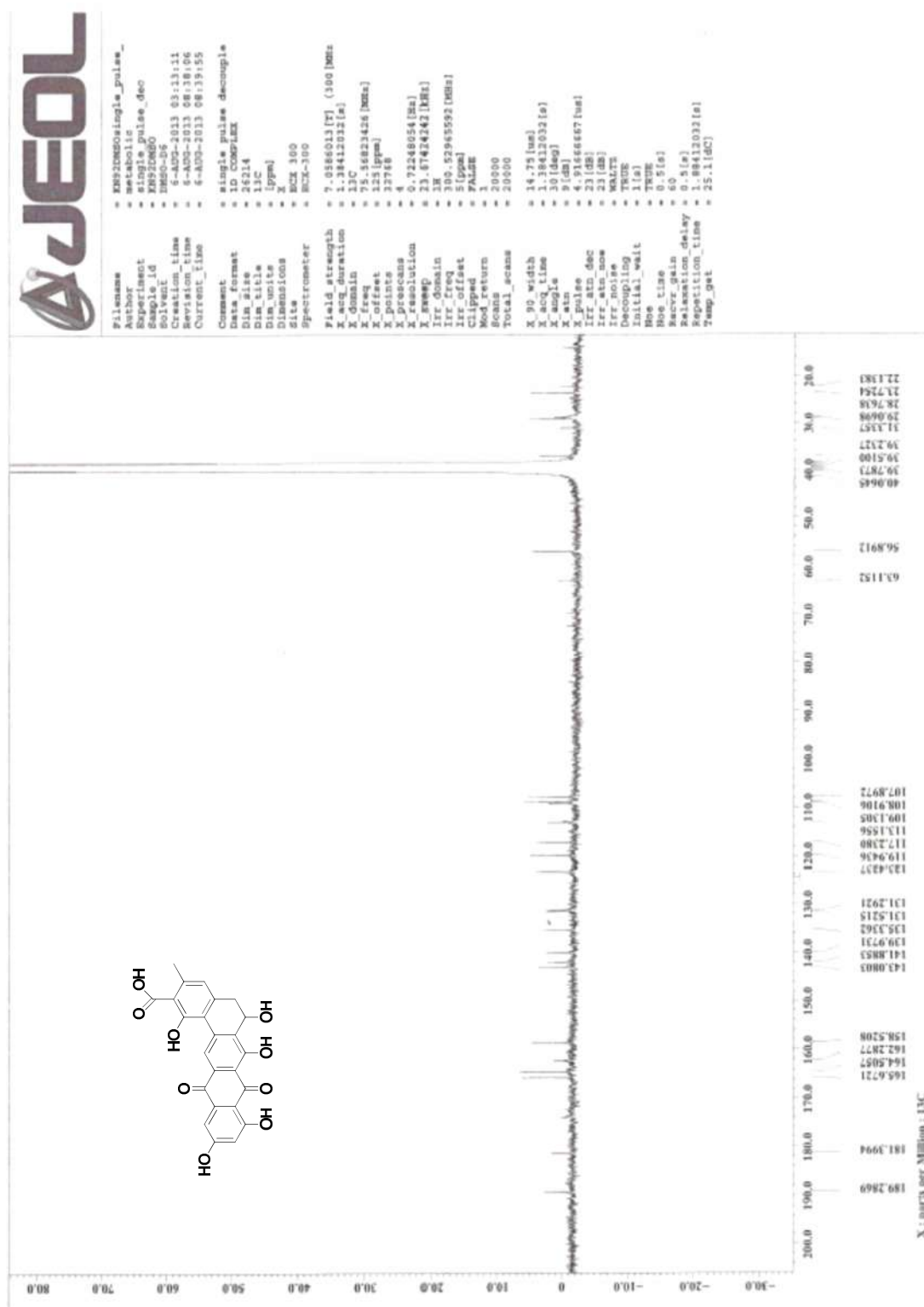


Figure 37. UV/Vis spectrum of compounds 4-11.

Figure 38.  $^1\text{H}$ -NMR spectrum of compound **8**.

Figure 39. <sup>13</sup>C-NMR spectrum of compound 8.

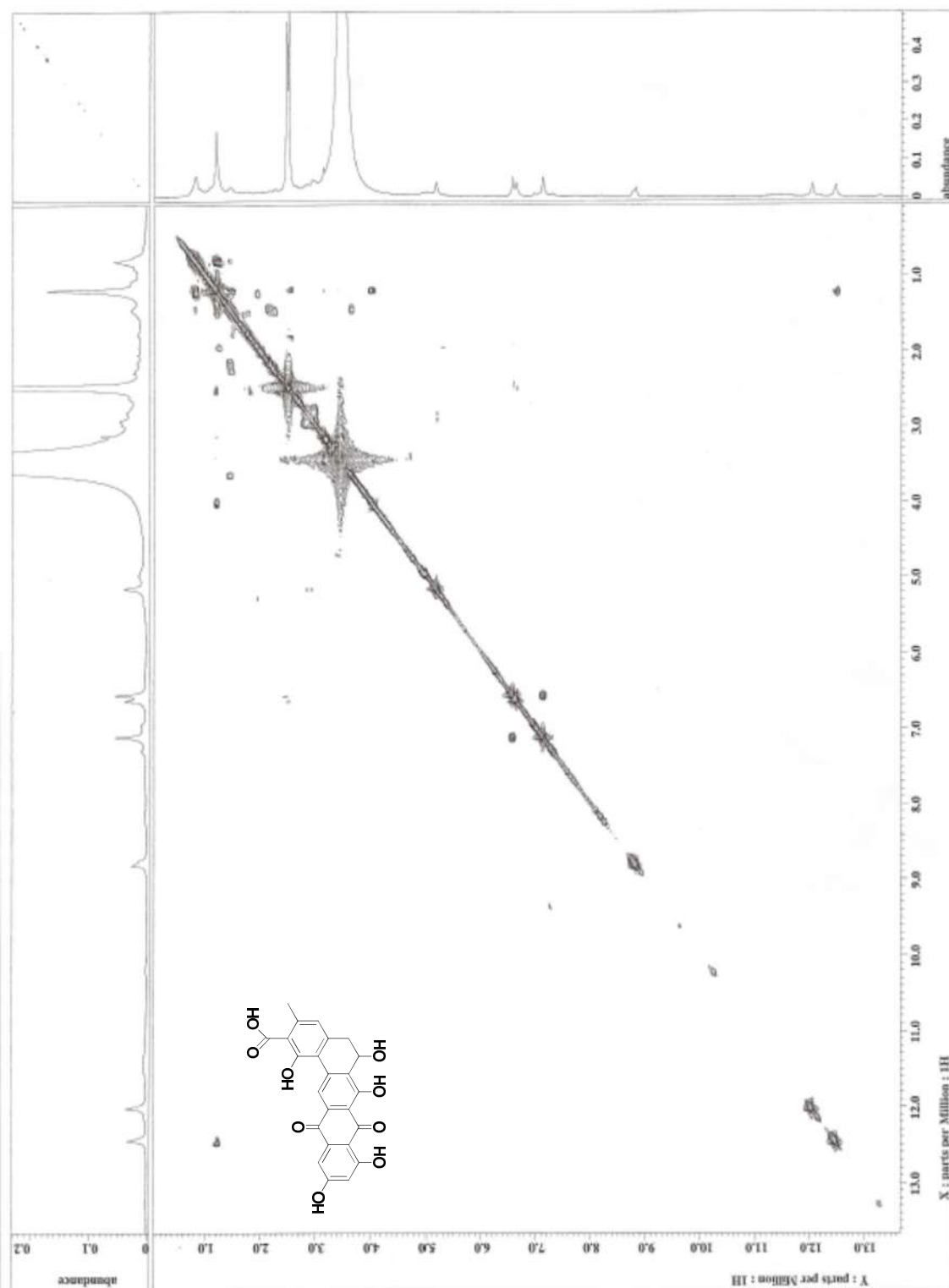


Figure 40.  $^1\text{H}$ - $^1\text{H}$  COSY of compound 8.

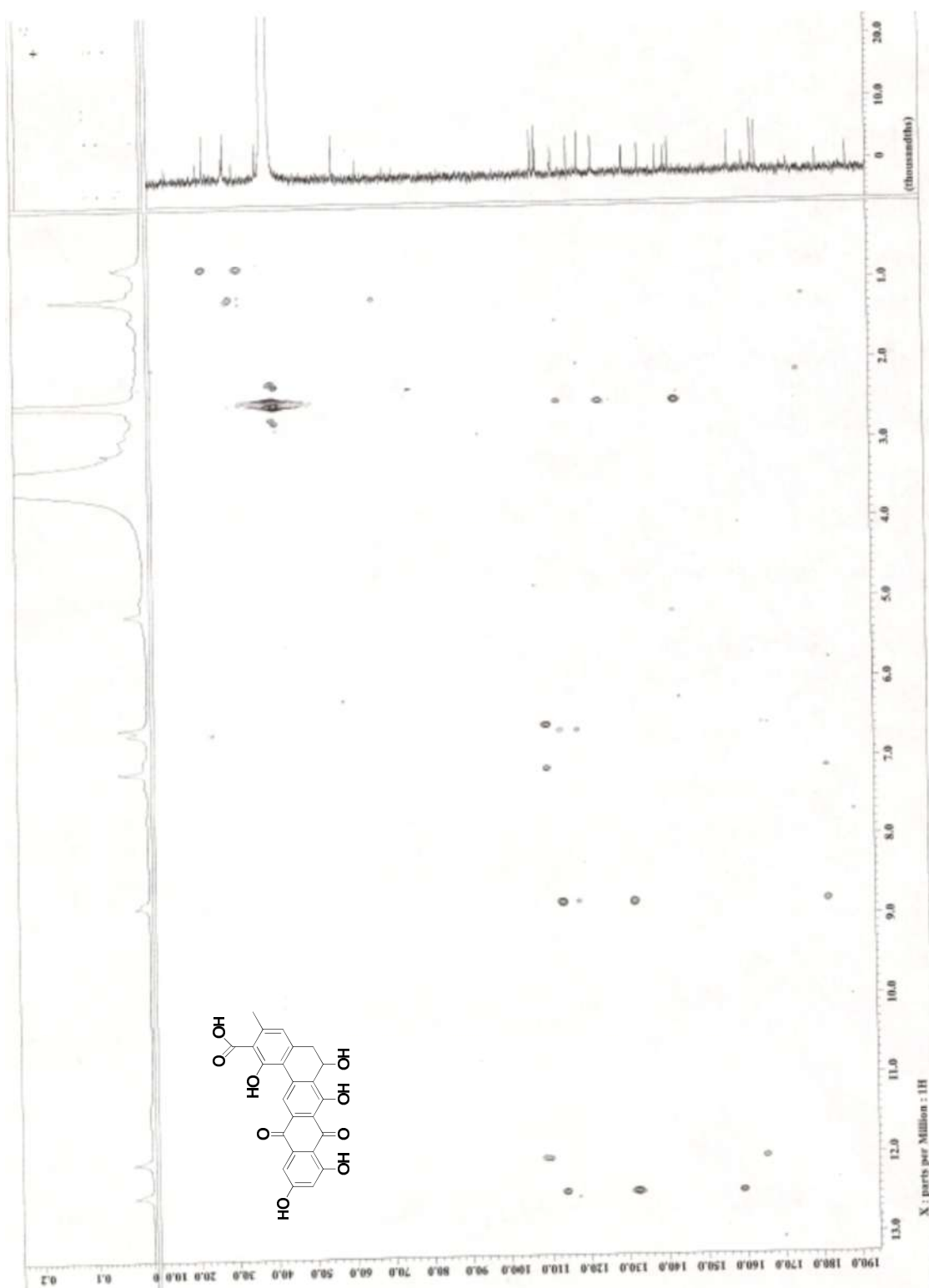


Figure 41. HMBC of compound 8.



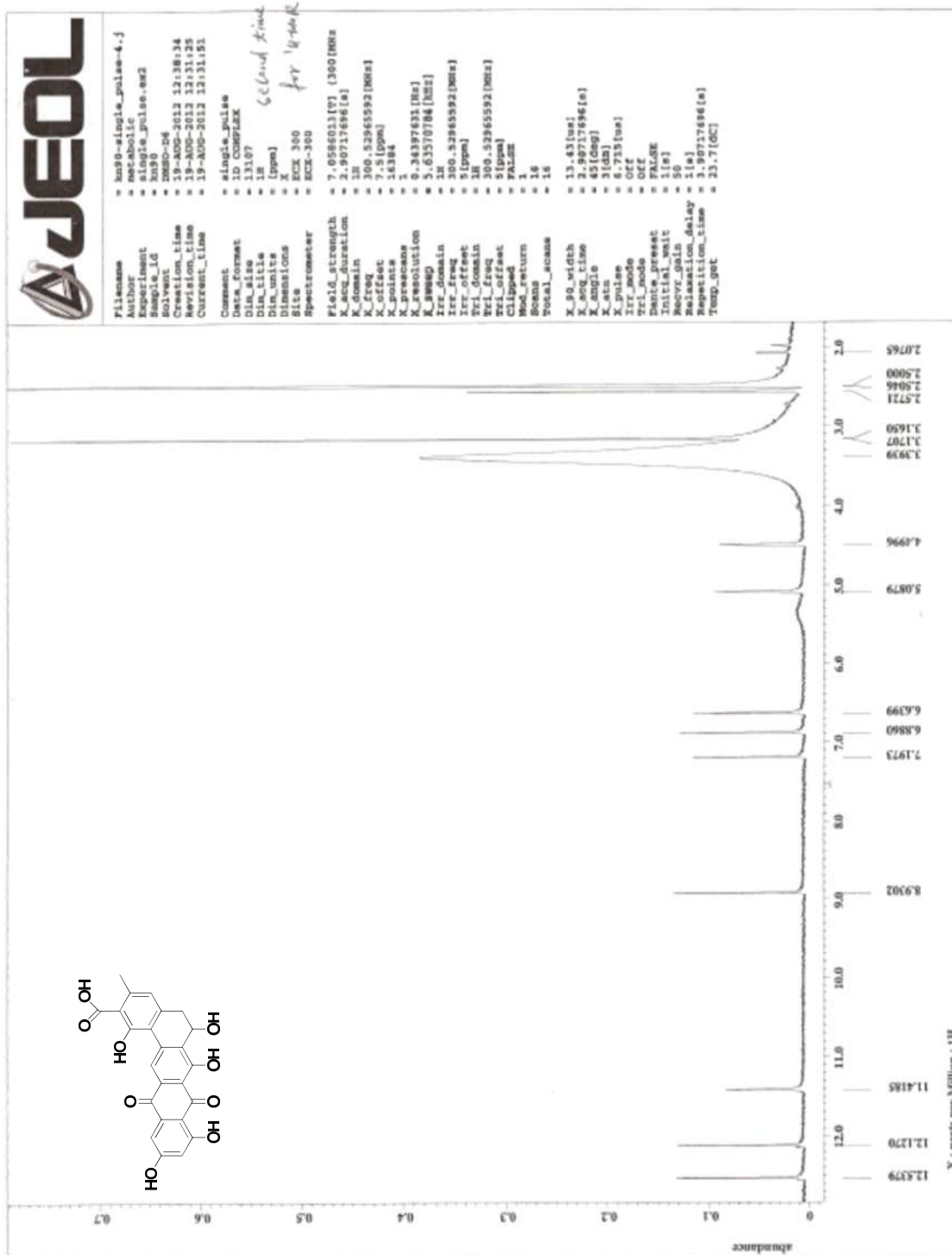
Figure 42. <sup>1</sup>H-NMR spectrum of compound 9.

Figure 43.  $^{13}\text{C}$ -NMR spectrum of compound **9**.

Figure 44.  $^1\text{H}$ - $^1\text{H}$  COSY of compound **9**.

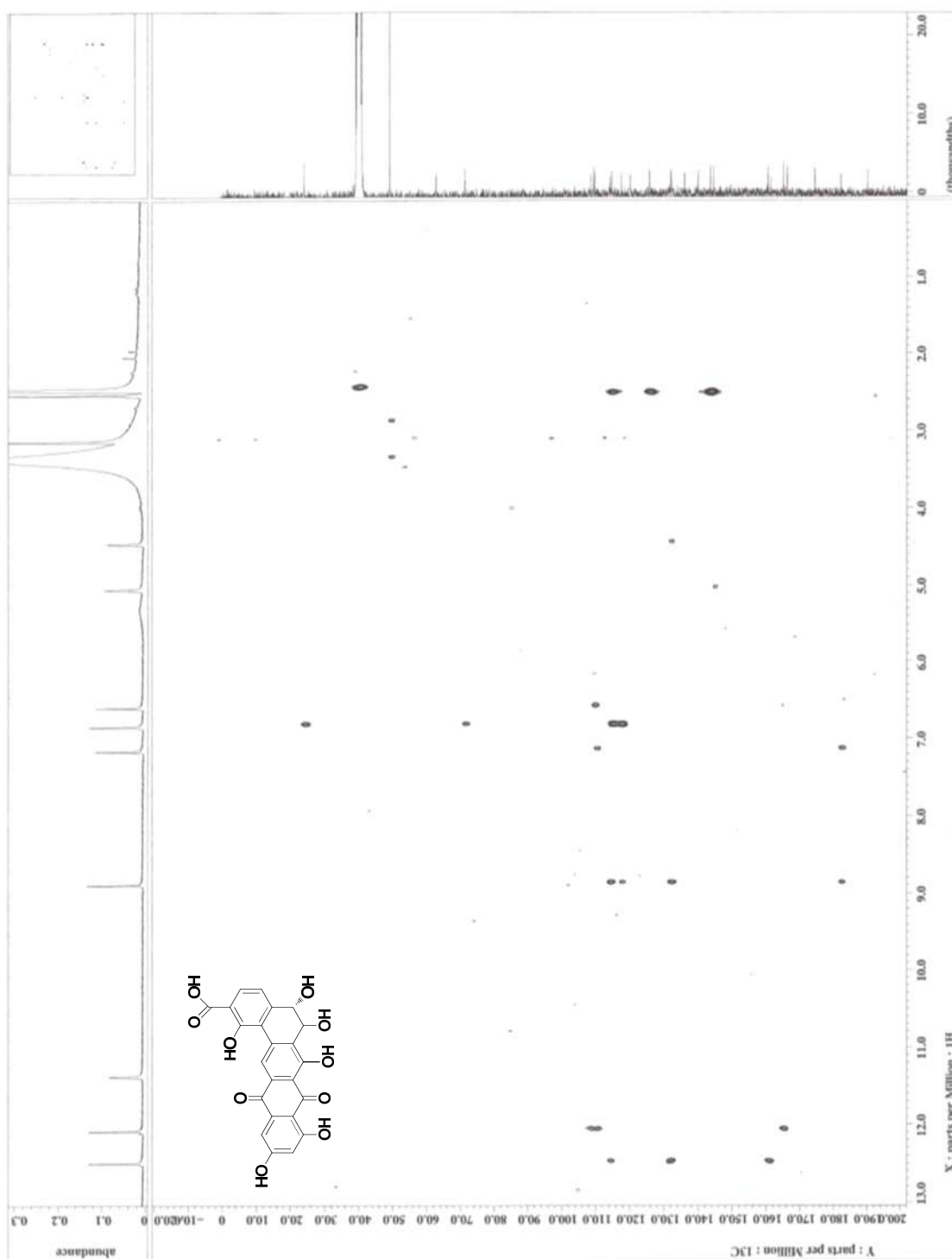
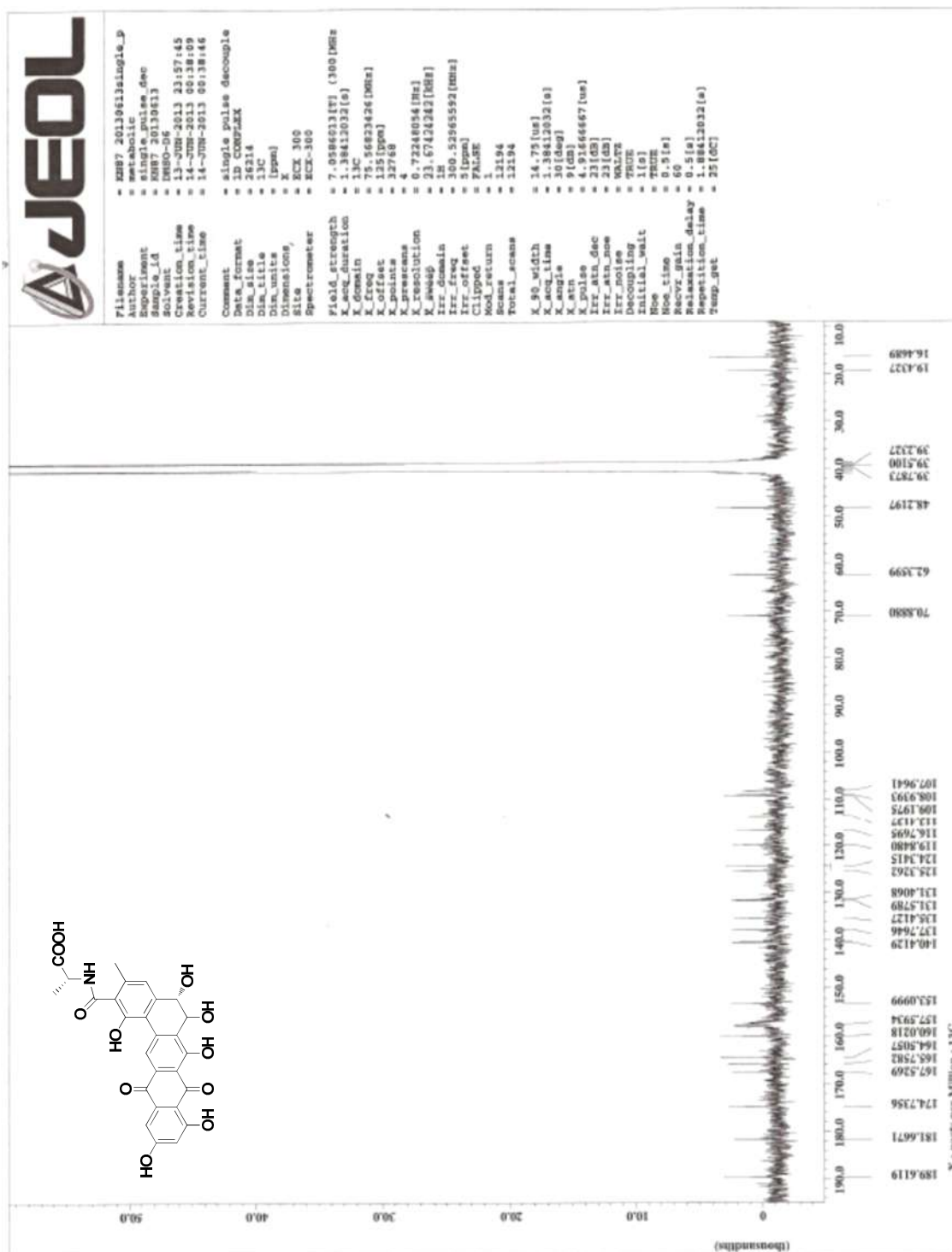


Figure 45. HMBC of compound 9.



Figure 47. <sup>13</sup>C-NMR spectrum of compound 11.

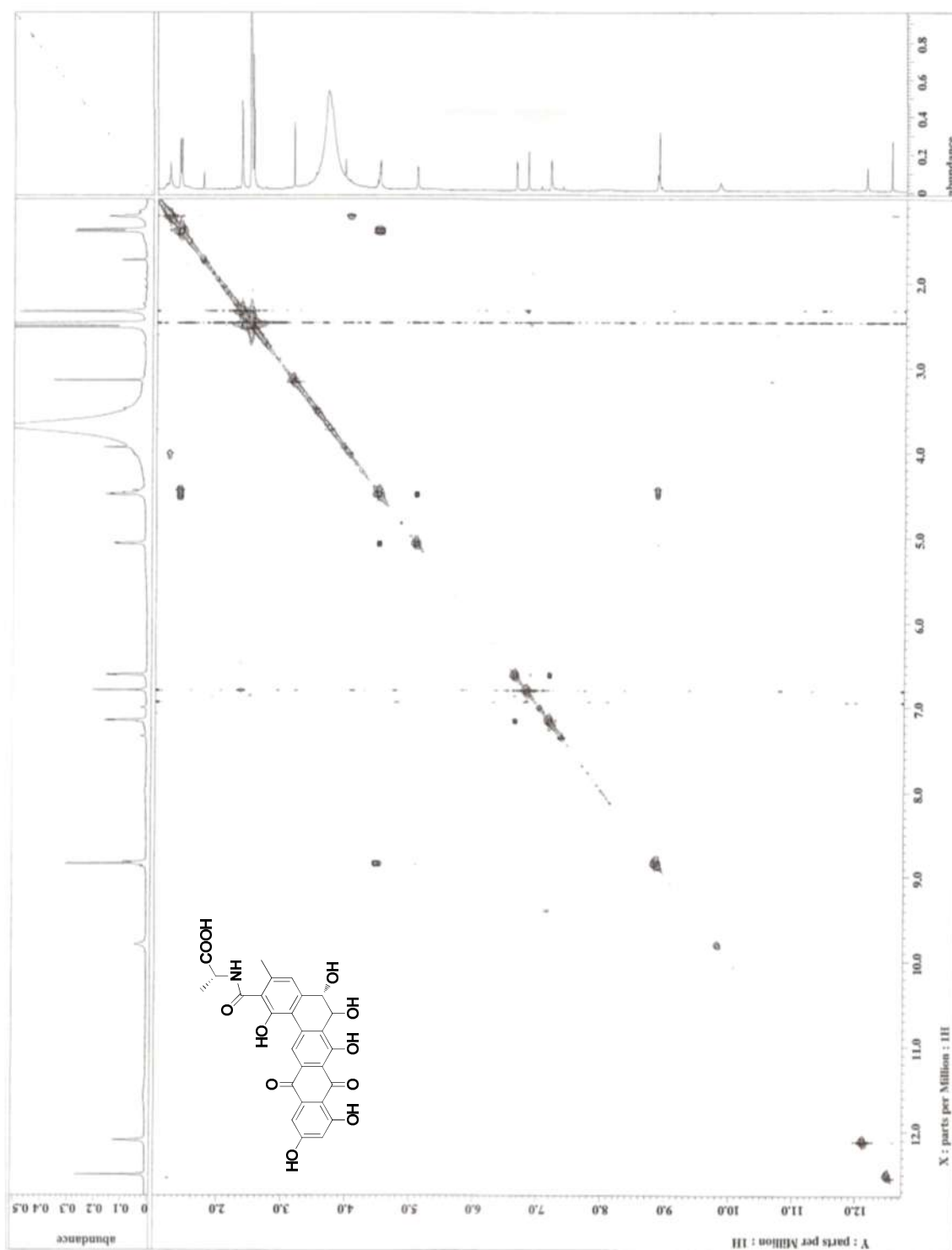
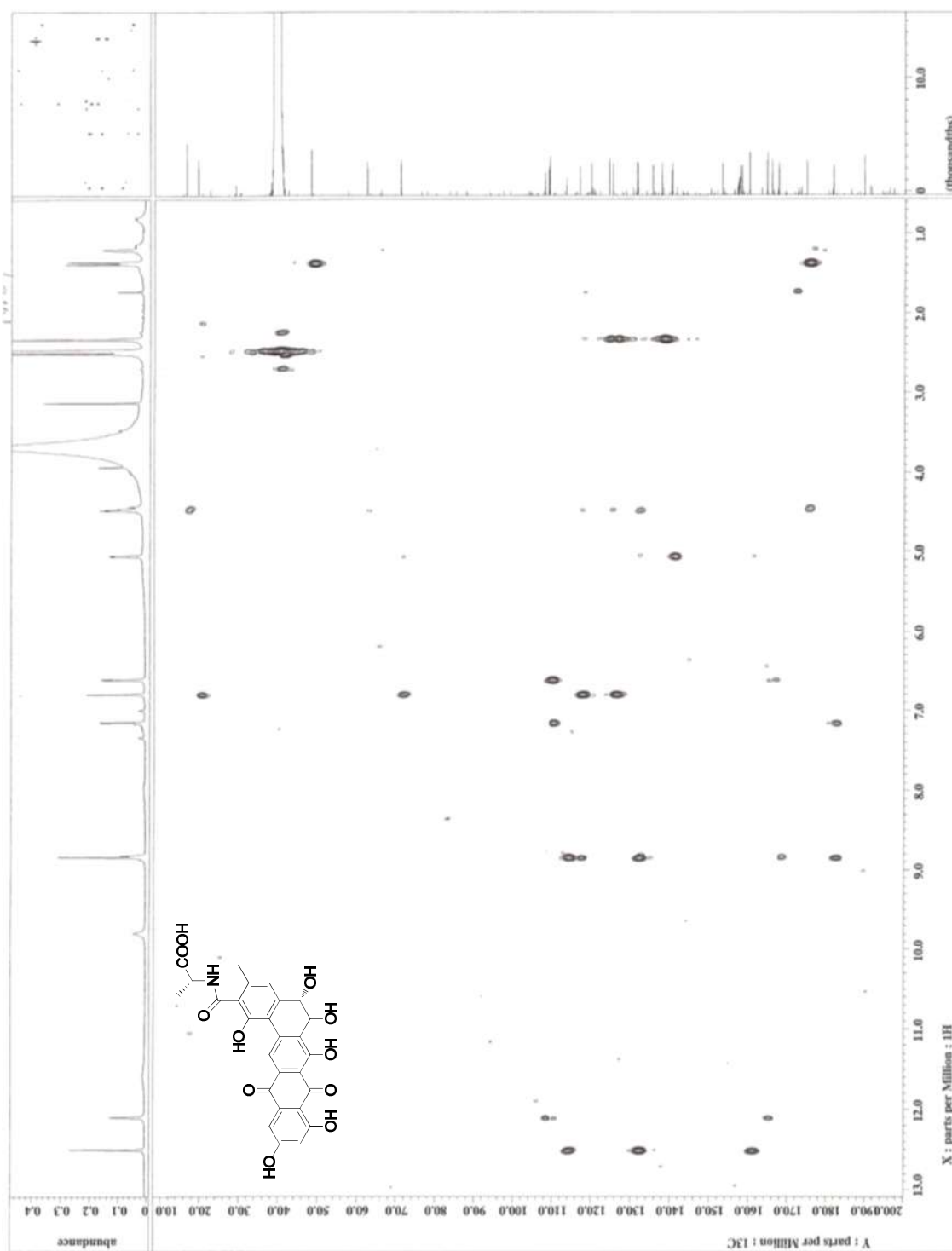


Figure 48.  $^1\text{H}$ - $^1\text{H}$  COSY of compound **11**.

Figure 49. HMBC of compound **11**.



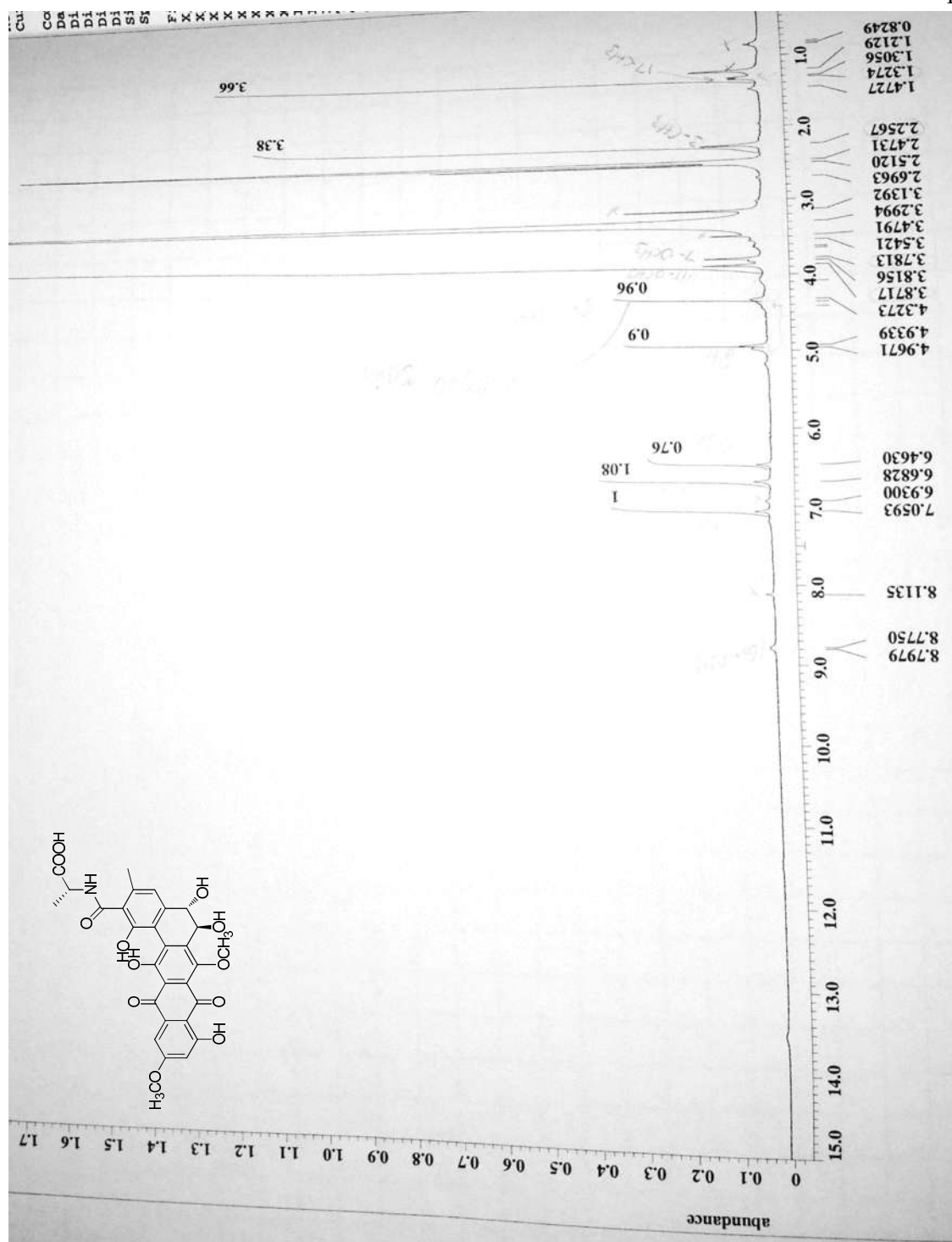


Figure 50.  $^1\text{H}$ -NMR spectrum of compound 14.

Figure 51.  $^1\text{H}$ - $^1\text{H}$  COSY of compound **14**.

## APPENDIX C.

### LC/MS analysis of **1-3** and **12**

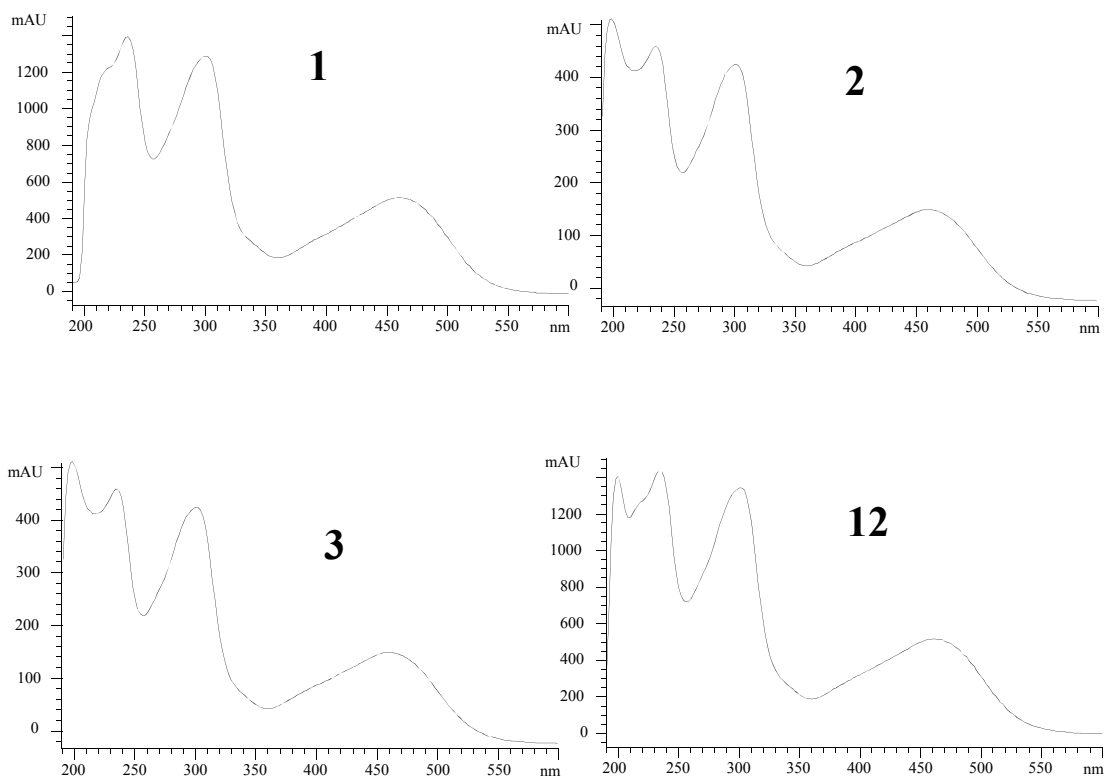
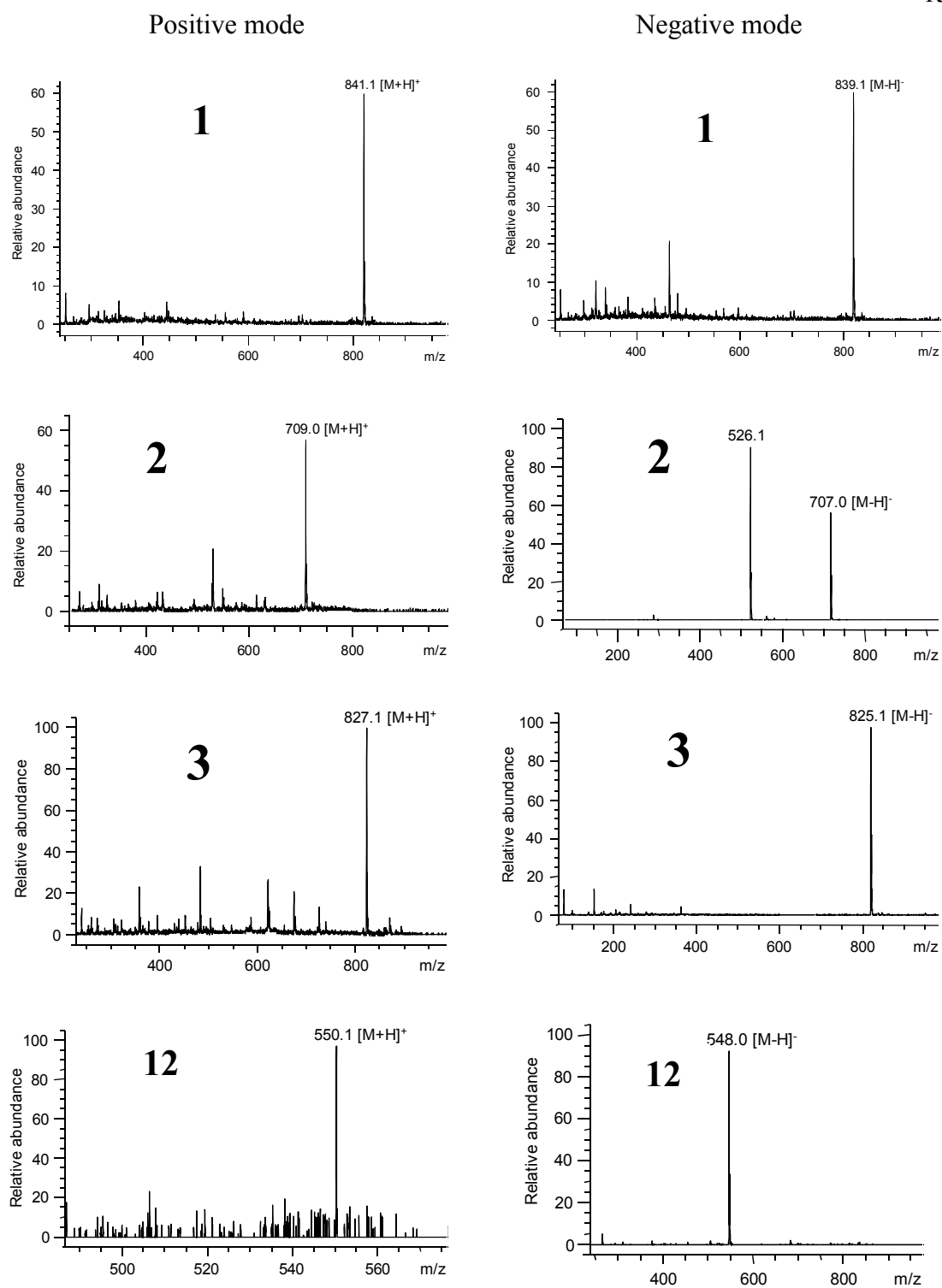


Figure 52. UV/Vis spectrum of **1**, **2**, **3**, and **12**.

Figure 53. ESI-MS spectrum of **1**, **2**, **3**, and **12**.VITA

## KANDY L. NAPAN

Metabolic Engineering Lab, Utah State University, 4105 Old Main Hill. Logan, UT 84322-4105

Contact: Email: kandy.napan@aggiemail.usu.edu, Phone: (385) 226-0827

- |                                       |  |
|---------------------------------------|--|
| <b>EDUCATION:</b>                     | <ul style="list-style-type: none"> <li>• <b>Ph.D. Biological Engineering</b>, May 2016<br/>Department of Biological Engineering, Utah State University, Logan, USA.</li> <li>• <b>B.S. Biology</b>, March 2010<br/>Department of Natural Sciences and Mathematics, Federico Villarreal University, Lima, Peru.</li> </ul>  |
| <b>PEER REVIEW PUBLICATIONS:</b>      | <ul style="list-style-type: none"> <li>• <b>Napan, K. L.</b>, Zhang, S., Anderson, T., Takemoto, J. Y., &amp; Zhan, J. (2015). Three enzymes involved in the N-methylation and incorporation of the pradimicin sugar moieties. <i>Bioorganic &amp; medicinal chemistry letters</i>, 25(6), 1288–1291.</li> <li>• <b>Napan, K.</b>, Zhang, S., Morgan, W., Anderson, T., Takemoto, J. Y., &amp; Zhan, J. (2014). Synergistic Actions of Tailoring Enzymes in Pradimicin Biosynthesis. <i>ChemBioChem</i>, 15(15), 2289-2296.</li> <li>• Bejar, V., Villanueva, F., Guevara, J. M., González, S., Vergaray, G., Abanto, E. <b>Napan, K.</b>, ... &amp; Vergaray, S. (2014). Epidemiología de las dermatomicosis en 30 años de estudio en el Instituto de Medicina Tropical Daniel A Carrión, Universidad Nacional Mayor de San Marcos, Lima, Perú. <i>Anales de la Facultad de Medicina</i> 75(2), 167-172.</li> <li>• <b>Napan, K. L.</b>, Zeng, J., Takemoto, J. Y., &amp; Zhan, J. (2012). A key cytochrome P450 hydroxylase in pradimicin biosynthesis. <i>Bioorganic &amp; medicinal chemistry letters</i>, 22(1), 606-609.</li> <li>• <b>Napán, K.</b>, Llanos, C., &amp; Espinal, C. P. (2010). Acute toxicity of Methomyl in <i>Poecilia latipinna</i> (Lesueur 1821)(Poeciliidae). <i>The Biologist</i>, 8(1), 21-28.</li> <li>• <b>Napan K.L.</b>, Zhang, S., Morgan, T., Takemoto, J. Y., &amp; Zhan, J. Specific Glucurodination of Pradimicin A Analogues by <i>Streptomyces rimosus</i> subsp. <i>rimosus</i> ATCC 10970. <i>In review</i>.</li> </ul> |
| <b>WORK &amp; INTERNSHIP HISTORY:</b> | <ul style="list-style-type: none"> <li>• Graduate research assistant, USU Metabolic Engineering Lab, Logan, UT – Aug. 2010 – May 2016.</li> <li>• Microbiology intern, Neoplastic Illness National Institute (INEN), Department of Microbiology, Peru – Jan. – March 2009.</li> <li>• Microbiology intern, Good Hope Health Center, Department of Pathology, Peru – Jan. – March 2008 and 2009.</li> </ul>   |
| <b>RESEARCH SKILLS:</b>               | <p><u>Metabolic Engineering Laboratory</u></p> <ul style="list-style-type: none"> <li>• Performed recombinant DNA technology: Molecular cloning in <i>E. coli</i> and <i>Actinomyces</i>, PCR, primer design, gel electrophoresis,</li> </ul>  |

DNA isolation, plasmid construction, transformation, protoplast preparation, and DNA sequence analysis.

- Worked with recombinant protein: Protein expression and purification from *E. coli* and *Actinomycetales*, SDS-PAGE, optimization of recombinant protein expression (solubility).
- Conducted enzymatic *in vitro* reactions and kinetics assays: Cytochrome P450, methyltransferase, and glycosyltransferase.
- Performed solvent extraction: Extract bioactive compounds from liquid and solid bacterial cultures.
- Purified second metabolites: TLC, HPLC, size exclusion, reverse phase, and silica column chromatography.
- Carried out antimicrobial susceptibility tests: Agar diffusion method and microdilution assay.
- Executed and evaluated analytical experiments: HPLC, LC-MS, GC, Spectrophotometer, and proton NMR for structure determination of small molecules.
- Researched the optimization of bacterial culture bioreactor for second metabolite production.
- Developed protocols and culture techniques.
- Technical report writing.

#### Management / Mentoring

- Developed management skills by training and directing 6 undergraduate researchers and leading research projects funded by NIH.
- Consulted and given technical guidance to co-workers and students in molecular biology techniques using self-optimized and standard protocols.
- Responsible for calibration and maintenance of analytical equipment including Eppendorf bench-top centrifuge, Beckman ultracentrifuge, and pH meter.
- Management of biological waste.
- In charge of supplying culture media, reagents, and restriction enzymes in the laboratory.

#### Bioinformatics and Computer Skills

- Genomic and proteomic analysis: BLAST and FASTA search analysis, multiple sequence alignment with DNASTAR 11, DNAssist, DNA 2.0, Frameplot 2.3.2, PKS/NRPS analysis Website and associated tools and databases.
- ChemDraw12.0.
- Office products.

#### **PRESENTATIONS & AWARDS:**

- Poster presentation in the 2015 SBI Annual Meeting, Logan UT.
- Poster presentation in the 2014 SIMB Annual Meeting, St. Louis, MO.
- RGS Graduate Student Award 2014.
- Poster presentation, 2nd place in the 2014 USU-UNLV Mini Symposium, Las Vegas, NV.

- Poster presentation in the 2014 SBI Annual Meeting, Logan UT.
- Podium and poster presentation, **Grand prize** in the 2013 IBE National Conference, Raleigh, NC.
- Podium and poster presentation, in the 2012 IBE National Conference, Indianapolis, IN.
- RGS **Graduate Student Award** 2012
- Podium presentation, **2nd place** in the 2011 IBE Regional Conference, Logan, UT.
- Poster presentation in the 2011 UBEC Annual Meeting, Salt Lake City, UT.
- Center for women and Gender (CWG) **travel award** 2012.
- Poster presentation, **3rd place** in the 2008 School of Biology, Lima, Peru.